PB-245 134

PROCEEDINGS OF THE FIRST MICROBIOLOGY SEMINAR ON STANDARDIZATION OF METHODS, HELD AT SAN FRANCISCO, CALIFORNIA, ON JANUARY 1973

S. S. Verner

Environmental Protection Agency
Washington, D. C.

March 1973

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275078

EPA-R4-73-022

MARCH 1973

Environmental Monitoring Series

PB 245 134

Proceedings of the First Microbiology Seminar on Standardization of Methods

Office of Research and Monitoring
U.S. Environmental Protection Agency
Washington, D.C. 20460

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SELECTED WATER RESOURCES ABSTRACTS

INPUT TRANSACTION FORM

1. Report No.

05A **W**

Proceedings of the First Microbiology Seminar on Standardization of Methods

5. Report Date

6.

8. Performing Organization Report No.

S. S. Verner, Editor-in-Chief

Office of Research and Monitoring
Environmental Protection Agency

13. Type of Report and Period Covered

12. Sponsoring Organization

Activity performed under Program Element 1H1327
EPA Report No. EPA-R4-73-022

This document contains the proceedings of the "Seminar on Standardization of Microbiological Methods" held in January, 1973. The Seminar brought together EPA microbiologists from all program elements, offices and Regions to discuss problems of mutual concern in methodology.

This Seminar was organized into four segments, viz., standardization processes as related to enforcement, research activities, and regional problems; microbiological parameters which consumed the major portion of the meeting; analytical procedures as related to sampling and quality control; and a final paper summarizing research requirements prior to standardization. In addition, the meeting was structured to permit free discussion of the topic parameter after each formal presentation and, where available, verbatum or summary discussions are presented following each respective paper in these proceedings.

17a. Descriptors

Microbiology in Aquatic Ecosystems, Aquatic Pathogens

17b. Identifiers

GPO

19. Security Class (Report)

20. Security Class (Page)

21. No. of Pages 222

22. Price 7.25/2.25

Send To:

WATER RESOURCES SCIENTIFIC INFORMATION CENTER
U.S. DEPARTMENT OF THE INTERIOR
WASHINGTON, D.C. 20240

EPA-R4-73-022
March 1973

PROCEEDINGS OF THE FIRST MICROBIOLOGY SEMINAR ON STANDARDIZATION OF METHODS

San Francisco, California

January 1973

Sponsored by

Quality Assurance Division
Office of Research and Monitoring
U.S. Environmental Protection Agency
Washington, D.C. 20460

For sale by the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402

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FOREWORD

The Seminar on Standardization of Microbiological Methods was organized to develop within the Environmental Protection Agency (EPA) common methodological techniques and to obtain broad Agency acceptance of a standardization program in microbiology. A meeting limited to EPA microbiologists was held to serve as a forum for airing common problems and by opening channels of communication among Agency microbiologists, help to clarify, unify and strengthen the standardization program.

These proceedings are not intended to present the Agency's complete standardization plan in microbiology. The Seminar was necessarily of limited scope and additional meetings will be held to complete the program. The Office of Monitoring welcomes comments on this publication as well as suggestions for future activities.

Willis B Foster

Willis B. Foster
Deputy Assistant Administrator
for Monitoring

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PREFACE

This document contains the proceedings of the "Seminar on Standardization of Microbiological Methods" held in January, 1973. The Seminar and these proceedings are representative of a continuing effort by the Office of Monitoring to highlight and alleviate, through Agency-wide cooperative efforts, areas and issues of monitoring requiring technical or administrative support.

This Seminar was organized to focus on priority problem areas and was divided into four segments, viz., standardization processes as related to enforcement, research activities, and regional problems; microbiological parameters which consumed the major portion of the meeting; analytical procedures as related to sampling and quality control; and a final paper summarizing research requirements prior to standardization. In addition, the meeting was structured to permit free discussion of the topic parameter after each formal presentation and, where available, verbatum or summary discussions are presented following each respective paper in these proceedings.

The publication of these proceedings will be followed by a compilation (compendium) of candidate methods based on the suggested recommendations of the authors. The compilation will be written by a committee of experts for each of the microbiological parameters and will serve as a data base of analytical methods in microbiology.

The Seminar brought together EPA microbiologists from all program elements, offices and Regions to discuss problems of mutual concern in methodology. The appendix lists the agenda for the Seminar and the names of those attending the meeting. It is anticipated that future Seminars will be held to complement this one by focusing on issues not considered here.

STANDARDIZATION PROCESSES

ENFORCEMENT ACTIVITIES

David I. Shedroff[*]

In order to present the role of standardization of microbiological sampling and analysis in Federal enforcement, it will be necessary to briefly describe the enforcement provisions of the new Federal water pollution control bill (see Figure 1), particularly as they affect the work of the microbiologist, and then describe how expert opinions of microbiologists concerning quantitative or qualitative presence of particular indicators or pathogens becomes admissible evidence in an administrative or court proceeding. Finally, some of the areas of impact of the new law and the methods of introducing evidence on further standardization of microbiological sampling and analysis will be related.

A. New Law

The Federal Water Pollution Control Amendments of 1972 (1) became law in October of last year. This statute strengthens Federal and joint Federal-State enforcement authority over dischargers and gives added responsibilities to microbiologists in the enforcement area.

1. Emergency Authority

For the first time Congress has given authority to the government to obtain a court order to immediately stop discharges which present "an immiment and substantial endangerment to the health of persons or to the welfare of persons where such endangerment is to the livelihood of such persons, such as the inability to market shellfish" (2). One of the means of proving health hazards will obviously be to prove the presence of pathogens. If past history of closure of shellfish beds gives a preview of possible use of the "endangerment of welfare" portion of this new section, part of the proof will often turn on the validity of results of analyses for total coliform, which are the basis for orders closing the beds and on proof of the sources of the coliform. Here is a prime example where EPA microbiologists may well have to serve as government witnesses.

2. Toxic Pollutants and Hazardous Substances

Closely related to the emergency provisions of the Act are those covering toxic pollutants (3) and hazardous substances (4). The Administrator will shortly publish his

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[*] Enforcement Division, National Field Investigation Center, Cincinnati

TABLE I. MICROBIOLOGICAL ACTIVITIES UNDER ENFORCEMENT AND RELATED PROGRAMS OF FEDERAL WATER POLLUTION CONTROL ACT, AS AMENDED

Section of Law	Activity Covered	Activity Description	Anticipated Activities for Microbiologists
504	Emergency Authority	Injunction may be issued on proof of "imminent and substantial endangerment to the health of persons or to the welfare of persons where such endangerment is to the livelihood of such persons"	Proof of presence of pathogens in waters and from particular source(s); and in the case of shellfish beds presence of and sources of indicators authorizing closure.
307(a)	Toxic Pollutants	Discharge limitations, including zero if materials designated as toxic taking into account "toxicity . . . persistence, . . degradability, . . presence of affected organisms, .. importance of affected organisms and the effect of the toxic pollutant on such organisms"	Identification and quantification of pollutants designated as toxic by Administrator (may include viruses)
311	Hazardous Substances	"Elements and compounds which, when discharged in any quantity. . .into waters,. . .present an imminent and substantial danger to the public health, including but not limited to, fish, shellfish, wildlife, shorelines, and beaches."	Performance of degradability tests and experiments under actual or simulated conditions
402 and 301(b)	Permits for publicly owned (municipal) treatment works	Attainment of "secondary treatment", as defined by administrator, or treatment necessary to meet water quality standards, whichever is more stringent by 1977, and "best practicable waste treatment technology over the life of the works" by 1983.	Analyses to determine compliance or non-compliance with microbiological portions of permit requirements.
402 and 301(b)	Permits for non-publicly owned (industrial) treatment works:		
	a) Existing Plants	Attainment of "best practicable control technology currently available", or water quality standards, whichever is more stringent by 1977, and "best available technology economically achievable" by 1983.	Analyses to determine compliance or non-compliance with microbiological portions of permit requirements
	b) New Plants	Compliance with "National Industrial Standards of performance" which for a particular industry "reflects the greatest degree of effluent reduction. . . achievable through the application of the best available control technology, processes, operating methods, or other alternatives"	
307 and 301(b)	Pretreatment standards for discharges by non-publicly owned enterprises into publicly-owned plants.	Compliance with standards of pretreatment by category or categories of sources from existing plants or new plants. Standards for both categories of plant must prevent by discharge through a publicly-owned treatment works of pollutants which "interfere with, pass through, or (are) otherwise incompatible with such works" Standards for existing sources will include time for compliance which may not exceed three years from date of promulgation.	Analyses to determine compliance or non-compliance with microbiological portion of the pretreatment standard.
405	Permits for disposal of sewage sludge	Permits required for disposal of sewage sludge (including removal of in-place sewage sludge from one location and its deposit in another location) where disposal "would result in any pollutant. . .entering. . .waters."	Analysis of sludges at time of transport and at disposal site to determine compliance or non-compliance with permit requirements.
312	Marine Sanitation Devices	Vessel sanitation devices must conform to performance standards issued by Administrator. New vessels must comply within two years of promulgation; existing vessels have five years in which to comply.	Analyses to confirm compliance with microbiological portions of performance standards. Analyses (for Coast Guard) to determine if device in place is operable in conformity with standards.
310	International pollution	Administrator may call hearing when he has reason to believe pollution is occurring from U. S. sources "which endanger the health or welfare of persons in a foreign country"	Microbiological surveys to determine if pollution being caused in a foreign country as aid to Administrator in deciding whether or not to call hearing and as evidence at hearing, if called.

initial list of toxic pollutants for which effluent limits, including zero discharge, will thereafter be set. After the limits are set by regulation, following public hearings, a violation of those limitations will subject the discharger to civil fine or criminal penalties, or a civil action to stop the discharge, but without the right to immediate cessation as in the case of discharges subject to the emergency provisions (5). Comparable enforcement provisions will be applicable to discharges of hazardous substances (6) after these substances are designated by the Administrator and the President specifies quantity limitations.

The statute contains technical definitions which define and limit what may be classified as toxic pollutants (7) or hazardous substances (8). Because the hazardous definition limits its applicability to elements and compounds, it is unlikely that living organisms will be included in that list; however, since penalties for violations depend on the severity of pollution (degradability of the substance) (9), testimony may be required describing results of experiments on degradability under actual or simulated spill conditions. The toxic pollutant definition specifically states that it includes "disease-causing agents" (10), so that organisms identified or identifiable by microbiologists may well be classified as toxic pollutants. To assure that viruses are included in the list of toxic pollutants, the Senate report on the bill specifically states "the presence of pathogenic organisms, including viruses" is one criterion for classifying a substance as toxic.

3. Permits for Discharges from Municipal and Industrial Plants

Perhaps the most sweeping reform of the new Act is in the point source discharge licensing system. Under the Act, municipal treatment plants and new and existing industrial plants discharging to any surface body of water must obtain a permit (12). All existing municipal and industrial plants must minimally attain "secondary treatment" and "best practicable control technology currently available," respectively, by July 1, 1977 (13). By 1983, municipal plants must attain "best practicable water treatment technology," and industrial plants must attain "best available technology economically achievable" (14). New industrial plants, when built must meet "national industrial standards of performance" (15), and plants discharging to municipal plants must conform to "pretreatment effluent standards" (16).

The Administrator must issue regulations defining each of the above terms within the next few months. The regulations on "best practicable treatment," "best technology," "pretreatment," and "required treatment" for new industrial sources will vary from industry to industry reflecting the nature of the wastes produced and the current availability of treatment methodology. Except in the case of pretreatment effluent standards, permits will then be issued requiring the discharges to conform with the requirements of the appropriate treatment requirements regulations, or to attain water quality standards (17), if that requires a higher degree of treatment than the 1977 requirement. These permits will place limits on discharges by parameters and will include a requirement of self-monitoring and reporting (18). Violation of permit conditions or failure to obtain a permit can subject the violator to stringent civil or criminal penalties whether the permit is issued by EPA (19), or by a State or Inter-State agency (20).

It is almost certain that the requirements of "secondary treatment" will require the inclusion of microbiological parameters in permits for municipal treatment plants, and that permits issued to industries where the presence of microbiological organisms is a valid indicator of the quality of treated effluent will include such parameters. Some of these industries are pulp and paper, tanneries, and the processing of food and dairy products, including sugar plants. The nature of wastes from manufacturers of pharmaceuticals will almost certainly require permits from discharges by this industry to contain one or more microbiological parameters. In addition, the proximity of a discharge to a watercourse for which there are microbiological standards may require the inclusion of microbiological parameters on other discharges, or the inclusion of more stringent numbers than those required under the treatment requirement regulations, e.g., discharges to or near water intakes, bathing beaches, and shellfish beds.

The primary source of information on whether or not there is compliance with permit conditions will be reports made by the discharger. The discharger's data will presumptively be admissible in court proceedings as "business records" (21).

4. Miscellaneous Provisions

The new bill has several other enforcement provisions which will bring microbiological testing into play. There is, for example, a special provision requiring persons depositing sewage sludge which might reach waterways to obtain permits (22). The regulations on issuance of such permits will no doubt include microbiological criteria.

Likewise, the performance standards for vessel sanitation devices (23) will undoubtedly include a maximum total coliform or fecal coliform criterion. In addition, the Act contains an absolute prohibition against discharge of biological warfare agents (24).

5. Abatement of International Pollution

Finally, the Act gives the Administrator the authority to call a hearing on alleged pollution in the United States believed to be endangering health or welfare of persons in a foreign country (25). The matter is heard first by a hearing board whose recommendations are transmitted to the Administrator for implementation. The recommendations may include suggesting the commencement of legal action. In any such legal action, the presentations before the hearing board are not automatically admissible as evidence, and such evidence and any later determinations must be admitted by the court. Microbiological evidence, e.g., relating to discharges from municipalities, beet sugar factories, and pulp mills can be expected to be part of hearings and trials under the section of the Act relating to International Pollution.

B. Admissibility of Microbiological Evidence

1. Courts, Agencies and Boards as Finders of Fact

Violations of the type described previously can usually result in the imposition of civil penalties by the Administrator, a hearing board, or a court; the imposition of criminal sanctions by a court, or the entry of a court order requiring a discharger to take or cease a particular action. Except for civil penalties imposed by the Administrator, it is clear that formal hearings will be required and that a final report as such will not be acceptable evidence without a satisfactory showing of authenticity (26). The Federal Rules of Civil Procedure define the form and admissibility of evidence in Federal courts, and the same general rules will probably apply to testimony before hearing boards. Rule 43 of the Federal Rules states, "In all trials the testimony of witnesses shall be taken orally in open court unless otherwise provided by these rules. All evidence shall be admitted which is admissible under the Statutes of the United States." Two of these statutes relate to exceptions to the hearsay rule. The hearsay rule states that generally persons may only testify to what they know personally, and that they must be subject to cross-examination.

2. Business Records and Government Records

a. Business Records

Under some circumstances business records are admissible without requiring the maker of the record appear. The Federal Act (27) says that such records may be admitted in evidence as the record of an act, occurrence, or event "if made in the regular course of . . . business and if it was the regular course of such business to make such memorandum or record at the time of such act . . . occurrence or event or written a reasonable time thereafter." Thus, the results of microbiological examinations may be admitted if there is a normal routine and it is followed. In order to establish that there is a set routine, it is wise to put it down on paper; as an example the simple sentence "after this date, all microbiological examinations will be made using appropriate directions as contained in part 400, 13th Edition Standard Methods" (28) may be used. Instruction sheets or operating manuals are used in some EPA microbiology laboratories at the present time. Since routines do change from time to time, it is recommended that all personnel sign dated receipts for the instruction materials as originally distributed and for modifications as issued from time to time. The statute also requires that the documents be prepared at or within a reasonable time after a particular occurrence. This suggests that it is a wise procedure to put all information directly into the log book or on bench cards rather than on scraps of paper and later copying over from these scraps of paper.

b. Government Records

Bench cards and other indicators of raw data may also be admissible as government records (29). Here again there is a requirement that there be an official record; the preparation of appropriate instructions defining examinations to be performed will be of value in getting the data admitted.

c. Limitations on the Admissibility of Business and Government Records

Although the statutes do not specifically cover the point, it is clear from the examination of cases under the two acts that one of the requirements for admissibility is that the document has inherent probability of trustworthiness. Thus, a trial judge has discretion in allowing or not allowing a document into evidence if there is doubt as to its trustworthiness (30). One criterion for the judge to consider is whether the particular analysis was done as a routine matter, or whether it was specifically done for or in anticipation of litigation (31). This is an indication of distrust of the situation, not of the individuals involved.

3. Direct Testimony as to Microbiological Analyses

Even where records may be admitted under the special acts described above, many government attorneys prefer to use direct testimony of the participants. They point out that there is a greater psychological impact involved when the trier of facts relates a particular event or datum to a person rather than to a piece of paper. This is particularly true where one side relies on documents and the other side uses convincing witnesses.

When testifying as an expert witness, you are in a position to give opinions as to the absence or presence of microbiological growths; most other witnesses are limited to stating what they themselves saw or heard. Rule 702 of Rules of Evidence for the U. S. Courts and Magistrates which will become applicable to all Federal trial courts regardless where located puts it this way:

> "Rule 702. Testimony by Experts: "If scientific, technical, or other specialized knowledge will assist the trier of fact to understand the evidence or to determine a fact in issue, a witness qualified as an expert by knowledge, skill experience, training, or education, may testify thereto in the form of an opinion or otherwise."

These rules have been promulgated by the U. S. Supreme Court and will become effective July 1, 1973 unless voided by Congress. Thus the expert microbiology witness must be in a position either on direct examination or on cross-examination to justify his conclusions in all ways. This includes both, the general acceptance of the test methodology and that the methodology was followed to the letter.

On a parallel plane, testimony on results using a particular method may be inadmissible as irrelevant to the issues of the lawsuit if the offense is described as including a particular test methodology and the stated method is not followed (32). The probable inadmissibility of MF results to prove a violation of an MPN limit in shellfish waters is an obvious example.

Assuming the correct methodology is used, the witness will still have to prove the validity and accuracy of his results. If the question concerns details of the particular analysis, the presence of documents showing who did what and when will obviously be of great benefit. Having such documents will often forestall cross-examination questions on how well you recall the details of a particular analysis when hundreds are performed over a period of time. Such documentation is also a great value in convincing a trier of fact that your result is more probably correct than the one testified to by the other party's microbiologist.

Of course, just being more accurate and precise does not mean that the trier will accept your results. How to project yourself as a witness is a topic you should discuss with your counsel or someone experienced in presenting legal testimony. Giving an opinion in a field outside your area of expertise and shown to be wrong impinges on the credibility of all of your testimony and should be avoided at all costs.

C. Conclusion

In closing, there are four areas of standardization to consider in light of enforcement requirements of the new law and the methods of translating data into evidence:

1. The area of standardization of equipment, reagents, media, and filters.

2. The area of defining the preferable method for a given type of sample--and having the yardstick included in a discharger's permit where required.

3. Modifying Standard Methods where presently published techniques are inadequate, e.g., in connection with effluents, particularly untreated or particularly treated effluents, viz., should procedures be changed to compensate for the effects of highly chlorinated effluents?

4. The area of defining standard tests for identification and quantification of pathogens which may be included in the list of toxic pollutants or which may become the subject of action under the emergency provisions of the Act.

REFERENCES

1. PL 92-500, 86 Stat. 816, 33 United States Code (U.S.C.) Sec. 1151 et seq.
2. Section 504 of Federal Water Pollution Control Act, as amended (FWPCA)
3. Section 307(a), FWPCA
4. Section 311, FWPCA
5. Section 309, FWPCA
6. Section 311, FWPCA
7. Section 307(a), FWPCA and Section 502(13) FWPCA
8. Section 311(b)(2), FWPCA
9. Section 311(b)(2)(B)(ii), FWPCA and Section 311(b)(2)(B)(iii), FWPCA
10. Section 502(13), FWPCA
11. Senate Report 92-414, P. 77
12. Section 402, FWPCA
13. Section 301(b)(1), FWPCA (Secondary treatment may be deferred until four years after approval of construction grants approved during FY 1975)
14. Section 301(b)(2), FWPCA; with economic exceptions, 301(c)
15. Section 306, FWPCA
16. Section 307(b), (c) and (d), FWPCA
17. Section 301(B)(i)(c), FWPCA
18. Section 402, FWPCA
19. Section 309, FWPCA
20. Forty Code of Federal Regulations (C.F.R.) Section 124.73, published in 36FR28399, Dec. 22, 1972

21. See Section B 2.a. of this paper

22. Section 405, FWPCA

23. Section 312, FWPCA

24. Section 301(f), FWPCA

25. Section 310, FWPCA

26. The regulations regarding public hearings under FWPCA prior to 1972 amendments are contained in 40CFR Part 106. This part requires testimony under oath and makes it clear that witnesses are subject to cross-examination.

27. Twenty-eight U.S.C. Sec. 1732

28. APHA, et al. Standard Methods for the Examination of Water and Wastewater, 13th edition, 1971. Where several alternative techniques are permissible with respect to a particular determination, the specific alternative(s) should be listed.

29. Twenty-eight U.S.C. Sec. 1733

30. LeRoy v. Sabena Belgian World Airlines, CA 2d, 344F2d 266, 1965; cert. den., 382 U.S. 878

31. cf. Hoffman v. Palmer, CA 2d, 129F2d 976, 1942; affd. 318 U.S. 109; rehearing denied, 318 U.S. 800

32. See U.S. EPA Water Quality Criteria Digest: (a) a compilation of Federal/State Criteria on Bacteria, August 1972 and (b) a compilation of Federal/State Criteria on Water Quality Sampling and Analytical Methods, August 1972.

OFFICE OF RESEARCH ACTIVITIES: MICROBIOLOGICAL METHODS

Louis G. Swaby, Ph.D.*

The current microbiological methodology research in EPA's Office of Research covers two program elements associated with (a) natural waters and (b) with water supplies including recreational waters. To some extent the methodology requirements of both programs are similar and the recent incorporation of the water supply research program into the Office of Research and Monitoring should result in a more efficient use of resources. A description of our ongoing work as taken from the official work plans follows.

Bacterial Methodology

This program is divided into two distinct areas: (a) methodology associated with the use of indicator organisms and (b) methods for the detection and identification of pathogenic bacteria.

Indicator Organisms

Water Supply Health Effects

(1) Evaluate and adapt existing techniques for the quantitative enumeration of total and fecal coliforms as indicators of fecal pollution to marine and estuarine waters.

(2) Evaluation of fecal streptococci as an indicator of fecal pollution in the marine and estuarine environments.

(3) Adapt existing techniques for Staphylococcus aureus to marine and estuarine waters for use as indicators of human health hazard associated with primary contact recreational activities.

Methods Development for Identification of Pollutants

(4) Improved methods for the detection of fecal streptococci.

(5) Investigation of the use of the fecal coliform test for industrial effluents.

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*Processes and Effects Division, Office of Research and Monitoring

The first two tasks are aimed at obtaining the information needed for establishing the traditional indicator organisms as indicators of fecal pollution in the marine environment. This is consistent with the emphasis on recreational waters and on estuarine waters frequently used for disposal of treated or untreated wastes. Little is known about the characteristics of coliforms and streptococci in the marine environment and it may be necessary eventually to look for more appropriate indicators of fecal pollution, for instance a chemical indicator.

The third task, of course, bears on the public health hazard in recreational areas. The choice of Staphylococcus aureus is associated with its high occurrence in the human environment, particularly the skin, and consequently it should be prevalent in recreational waters used for swimming.

The significance of the fourth task is obvious. An important aspect of the routine use of indicator organisms is to have rapid and accurate methods for the detection and quantification of the organism.

The fifth task is an extremely important one. Many, if not all, water quality standards include a fecal coliform standard. The permit program requirements also includes a fecal coliform measurement and it is quite likely that the effluent guidelines and standards will also include such a measurement. Yet, there is some question concerning its applicability to certain effluents or to waters receiving these effluents. In some cases regrowth of the coliforms has been noted and in other cases one might expect the opposite, a very rapid die off due to toxic substances. In either case, the significance of coliform measurements would be doubtful and challengeable.

Pathogenic Organisms

(1) Establish the potential pathogenicity of bacteria that proliferate in the marine and estuarine environments.

(2) Adapt existing quantitative techniques, for the organisms identified in the first task, to marine and estuarine waters.

(3) Investigate and evaluate cultural, biochemical and serological methods for Salmonella.

(4) Evaluate and adapt existing qualitative and quantitative procedures for Salmonella.

(5) Investigate fluorescent antibody - membrane filter techniques for enteropathogenic Escherichia coli, Salmonella and other pathogens.

(6) Develop the reverse phage technique.

The first two tasks at present emphasize Klebsiella which normally occurs in humans and can cause severe infections. These Klebsiella-caused diseases are very difficult to treat and in one Klebsiella-related disease mortality is still as high as 50% inspite of antibiotics.

The next two tasks are aimed at evaluating and improving the methods for Salmonella. The first will investigate enrichment media, biochemical confirmation procedures and serological methods. The results should be the definition of the most useful approach to the identification of Salmonella in water. The second task emphasizes the quantitative aspects of Salmonella methodology and will investigate both MPN and membrane filter techniques.

The fifth task is an investigation of the fluorescent antibody - membrane filter technique for enteropathogenic E. coli. The initial effort will evaluate commercially available antibody preparations made primarily for clinical application. Hopefully these preparations will prove useful and allow the development of a valuable technique for the water related problem.

The last task looks at a less well known method. The aim is to develop a screening technique which will identify the more prevalent pathogenic bacteria in a water sample. The technique involves plating a water sample mixed with phage specific for the bacteria of interest. The presence of the bacteria in the water sample will be indicated by clear plaques where the phage have lysed the bacteria. The technique should be rapid and easy to use and there is some possibility that it can be made quantitative.

Viral Methodology

The research on viral methodology is conducted under the same program elements as the bacterial methodology research. The emphasis is on methods to concentrate and recover the viruses in large volumes of water. The present tasks are:

(1) Survey water supplies in different geographical regions for the occurrence of viruses.

(2) Investigate, improve or develop viral methodology.

(3) Quantitative detection of small quantities of viruses in large volumes of water.

(4) Quantitative recovery of viruses from solids in water.

(5) Preservation of field-concentrated virus samples during transit.

The first task is for the purpose of defining the scope of the viral problem in water supplies. The information obtained will be of value in epidemiological studies, in establishing adequate treatment procedures and for identifying the detection techniques suitable for use in monitoring water supplies.

The second and third tasks, which represent the major portion of the viral research program, are aimed at developing, evaluating and improving methods for sampling all types of water for viruses. Viruses occur in water most frequently at concentrations less than one particle per gallon. To detect, identify and quantify requires that, at lease in some cases, the viruses in hundreds of gallons of water be removed and then recovered in appropriately small volumes of suspending media.

Several methods of recovering viruses from large volumes of water are being studied. Ultrafiltration is being investigated and so far does appear to have some applicability. The major difficulty of ultrafiltration is its inability to handle large volumes of water in a reasonable time. This project should be completed in the near future. This approach will not be the answer for all situations; however, it may prove applicable as a final concentration procedure. The use of absorbants is also being investigated. Of particular interest are the polyelectrolytes which have been shown to be very efficient for recovering poliovirus from water. For other viruses, recovery has been generally very low and the present effort is aimed at finding the conditions for increased recovery of these viruses.

The Melnick-Wallace Sampler[*] developed under this program is presently undergoing initial field evaluation which should be completed in the near future. This device is capable of processing 300 gallons of water per hour and is adaptable to heavily polluted as well as to relatively clean waters. If the initial evaluation is successful we will undertake a complete evaluation which will include the recovery of viruses from marine and estuarine waters.

In the fourth task attemps are being made to solve the problem of detecting viruses which are adsorbed to solids. A true assay of viral concentrations must include adsorbed viruses unless it can be shown that these viruses are never available for infection.

Lastly, regardless of the method of sampling, the samples must be returned to a virology laboratory to be assayed. The fifth task seeks a method of preventing loss of viral activity while the sample is being transported to the laboratory.

The research activities just described are recognized as minimal. Increased funding would allow the exploration of new approaches in addition to the refinement of traditional methods.

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[*] Water Research, Vol. 6, PP 1249-1256 (1972)

REGIONAL CONCERN AND ACTIVITIES
RE: STANDARDIZATION OF MICROBIOLOGICAL METHODOLOGY

Kathleen G. Shimmin*

A major Regional concern has been to demonstrate a coordinated approach toward analytical methodology whenever one of the various segments of the EPA Organization is dealing with other Federal agencies and States. There is the continuing problem of interpreting and effecting Headquarters' directives, as well as Regional, and yet appearing to remain consistent.

Consistencey of Methodology

It is essential to use the same approach, not a number of widely divergent paths when dealing with other Federal agencies and the States within the Region. If inconsistencies were to become apparent, they would serve as a cource of irritation to other agencies and render them less than cooperative.

One example of how this has recently been a problem, as in the field of chemistry, has been EPA's concern with the Corps of Engineers and EPA's national dredge criteria. The Corps spends millions of dollars on dredge projects, with increased expenses being incurred if open water disposal of the dredge spoils is prohibited. However, the criteria which were issued nationally did not contain methodology which was applicable for all the waters covered by the criteria. Thus, the very real possibility exists that the level of a given parameter analyzed and interpreted in one Region might be significantly different from that in another, even if the true values were similar. This means that a Corps District in one EPA Region might be treated quite differently from that in another Region - all because of a difference in methodology and interpretation.

With consistent methodology results and precedents from one Region can be applied to another. Data can be interchangeable (which is currently not possible - even with STORET data). The possibility of applying court decisions from one area to another becomes increased. Pollution originating from a State in one Region and impinging upon waters of a neighboring State in another Region would be identified by means of the same (or equivalent) methodology.

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*Laboratory Support Branch, Region IX

With the creation of EPA, many agencies were consolidated into one organization. Two of these agencies were Water Supply (Public Health Service) and Federal Water Quality Administration (FWQA). With the consolidation came methodology from each of these two organizations - a situation which is still in the process of resolution. However, this could creat a potential difficulty if both the Water Supply and former FWQA deal with a given State and give conflicting advice. Furthermore, if methodology were consistent, both groups could use the same quality control program. At the present time, these two former agencies each have separate ongoing analytical quality control programs.*

Improvement of Methodology

With a standardized approach, mechanisms for round-robin testing of methodology could be employed in a fashion similar to that used in inorganic chemistry. Problems such as interference from local water would come to light. These difficulties might not surface otherwise.

With a concerted effort toward methodology standardization a common basis for discussion would be established. In this way, microbiologists from all the programs within the Agency could have a chance to express their views and to modify methods which were inadequate. Without standardization, lack of uniform guidelines could lead to adoption of less desirable methods.

Methodology Standardization

It has been stated that established methods might be very slowly changed and would not keep up with current thinking and research. Therefore, a mechanism must be built into the standardization process to insure updating at an acceptable frequency.

Within a Region there might be a situation in which the standardized method might not apply. Mechanisms for alternatives should exist. For example, in Region IX there are areas in Micronesia which, if they have electricity at all, have an insufficient power supply. This means that at times the elevated-temperature fecal coliform tests cannot be run because the power supply is not consistent enough to provide a reliable 44.5 ± 0.2° C.

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*All EPA analytical quality control programs have been unified under the Quality Control Program guidelines approved by the Deputy Administrator on February 13, 1973.

However, there is a concomitant fear that if alternative methods exist, the wrong one may be chosen. This is a matter of competency of the laboratory. With the standardization effort, round-robin testing and exchange of information should reduce this problem.

It is essential that there be local Regional involvement in method evaluation. Often the Headquarters or other centralized administration are not aware of necessary modifications required by local situations. When this concept is ignored, disastrous conditions can arise. The case of EPA's original dredge criteria being based upon levels observed in fresh water in the Great Lakes Area and then being applied to the marine waters of other geographical areas is a familiar example.

Regional Standardization

In addition to intra-laboratory procedures, standardization activities of Region IX have been directed primarily towards training State and other Federal agencies to perform microbiological testing, quality control, and field studies by methods compatible with those used in the Regional Laboratory. Training has been conducted for the State of Nevada, USGS, and Micronesia. In the spring of this year, special courses will be given to the State of Hawaii and to the Navy. This approach has proven to be very useful for Region IX. It seems timely now for all the Regions and laboratory centers to develop appropriate strategy to deal with the problems of consistency of procedures and improvement of methodology.

MICROBIOLOGICAL PARAMETERS

TOTAL COLIFORMS

Harold L. Jeter*

Introduction

It must be assumed that all participants in this seminar have, within the past few weeks--or days--studied the bacteriology portion of the current (13th) edition of Standard Methods. Therefore, this will present an overview of the methodology for total coliforms, not so much in terms of a detailed account of step-by-step procedures as in terms of identifying the nature and number of options that are available in the testing procedures. From its first edition in 1905, Standard Methods has included tests for the bacteriological quality of water. Attention was not directed to the group now called "coliforms" until the third edition, which appeared in 1917.

Standard Methods

A terminology assigned to the coliform group has been somewhat variable, though the nature of the tests makes it clear that the tests are applied to the same group of microorganisms. The term "coliform group" has been used since the appearance of the 9th edition, in 1946. Prior to that time the group was termed the "coli-aerogenes group" in Standard Methods editions which were current between 1925 and 1946 (6th through 8th editions). Prior to that, tests for these organisms were covered under the term "Bacterium coli group" in the third through fifth editions, which were current between 1917 and 1925.

A definition of the coliform group has remained relatively stable through the various editions of Standard Methods. Prior to 1955 the text read "It is recommended that the coliform group be considered to include all aerobic and facultative anaerobic Gram-negative nonspore-forming bacilli which ferment lactose with gas formation." With the 1955 (10th) edition of Standard Methods the coliform group definition was amended to read "The coliform group shall include all of the aerobic and facultative anaerobic Gram-negative nonspore-forming bacilli which ferment lactose with gas formation within 48 hours at 35°C." With minor work changes this definition has been continuous to the present edition.

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*National Training Center, MDS, WPO

With acceptance of membrance filter methods for coliform tests, the Standard Methods Committee introduced a supplemental definition of coliforms, as applied to membrane filters: "In the membrane filter procedure, all organisms that produce a dark (purplish green) colony with a metallic sheen in 20 ± 2 hr. of incubation are considered members of the coliform group ---." Some refinements of this definition have been necessary; the current (13th) edition of Standard Methods says "All organisms which produce a colony with a golden green metallic sheen within 24 hr. of incubation are considered members of the coliform group ---."

Analytical methodology accepted as "standard" in Standard Methods comprises two different groups of procedures; these are the multiple tube and membrane filter methods.

Far from offering a choice of only two methods, each of these approaches to total coliform determination comprises a system of methodology with several identifiable options related to the extent to which the test is to be carried, media, and inoculation patterns. The result is that any one of a wide range of methods or "protocols" may be selected.

For example, consider the multiple tube method. The worker has a choice as to whether he will perform a Presumptive Test, a Confirmed Test, or a Completed Test. For the Presumptive Test there are two choices of media standard lactose broth or lactose lauryl sulfate tryptose broth. Thus there are two different methods for performing the Presumptive Test.

If the Confirmed Test has been selected, it is conducted as a continuation from the Presumptive Test. The Confirmed Test offers a choice of three different media - brilliant green lactose bile broth, eosin methylene blue agar, or Endo's agar. Based on the two identified Presumptive Test Methods and the three Confirmed Test media, there are six ways whereby a sample examination could be carried to the Confirmed Test.

Continuing this line of reasoning it is demonstrable that the six variants on the Confirmed Test would lead to a total of 8 different methods for the Completed Test of Standard Methods. Adding the variants of each, there are 16 ways in which coliform tests could be conducted by the multiple tube method.

To this, add the further complication of the number of different available tube inoculation patterns (six are shown in Standard Methods) and the number of different available protocols for total coliform determination soars to 96.

In fairness to the authors of Standard Methods, the number of available options recommended for a particular purpose is considerably lower than the above total. For example, for potability tests of drinking water, only the 5-tube test is designated by the authors as standard. Furthermore, in drinking water potability testing there is no provision for terminating the analysis at the Presumptive Test stage if positive tubes are found. The examination must be continued to the Confirmed Test or to the Completed Test stage. Thus there may be a total of approximately 14 different analytical pathways for drinking water testing. Similarly, for water of other than drinking water quality, in which quantitative data are desired, the authors of Standard Methods have reserved their recommendations to an inoculation scheme of 5 tubes in each of 3 or more sample volumes decreasing by decimal increments. The alternate use of 3 tubes for each sample volume is acknowledged but is not strongly recommended. Thus, for water of other than drinking water quality, a worker might have to select from 32 different test protocols.

The number of variations in standard membrane filter procedures is not nearly so great. There is a choice between direct incubation on the differential Endo-type medium or a preliminary enrichment on lactose lauryl sulfate tryptose broth; and there is a choice of whether a liquid or an agar-solidified version of the Endo-type medium should be used. Thus there are four variations on the standard membrane filter test for total coliforms.

In a separate section, under the definition of coliforms as recovered on membrane filters, a procedure is given for verifying colong interpretation on the filter. In effect, this comprises a Confirmed Test on individual colonies selected for verification of interpretation.

Prior to the 13th edition of Standard Methods acceptance of membrane filter methods was conditional. Acceptance required demonstration of suitability of the membrane filter method by each user laboratory, through a series of parallel tests of the membrane filter method and multiple tube method for the waters being tested. With the 13th edition this requirement has been softened and presently stands only as a recommendation.

Recommendations

1. It is recommended that EPA develop a manual of its own official methods for microbiological examination of environmental specimens. Let it be clearly noted that this does not constitute recommendation of any proposal to abandon Standard Methods in favor of such an agency manual.

2. As applied to tests for total coliforms, a recommended agency manual of microbiological methods would --

a. To the extent possible, identify the purposes of all total coliform tests which the manual is intended to encompass;

b. Sharply reduce the number of options in tests for total coliforms in environmental specimens;

c. On the basis of the identified purpose of total coliform tests, stipulate the normal first-choice agency method;

d. Describe all test procedures in simple language, in step-by-step fashion;

e. Supplement each test description with graphic representation of the test protocol. Do not show optional test procedures within any one graphic representation of a test protocol;

f. Provide for necessary departures from the designated procedures through stipulation of the nature of supportive evidence demonstrating need for such departures, and which demonstrate that the introduced substitution is better suited to the purpose at hand. It is here emphasized that professional judgment alone is not sufficient grounds for departure from standardized procedures. Hard evidence should be provided in any such case; and,

g. Provide a basis for professional consultation with qualified personnel within or without the Agency in matters not covered by the gency's manual of methods.

3. In my view, an EPA manual should not --

a. Designate as agency official method any procedure which is contradictory to, or not included in Standard Methods.

This does not imply that the Agency should avoid research into new methods, it is perhaps better-qualified and better-equipped to conduct such investigation than any other agency. However, it should not unilaterally try to impose its own agency methods on the field as a whole. The rational method here is to work within the Standard Methods system, and seek orderly introduction of new methods and acceptance only after adequate testing and evaluation by such laboratories as the Standard Methods Committee leadership may prescribe. This undoubtedly opens the door to the entire question of the interrelationships between this Agency and the Standard Methods leadership.

b. Seek to achieve brevity for its own sake, and particularly, it should not seek to achieve brevity at the expense of clarity; and finally, the manual should not --

c. Be the product of only a few microbiologists in the Agency. All major installations and organizational elements of the Agency which conduct total coliform tests of environmental specimens should be represented on any group developing such a manual of methods. Ratification of such a body of methods should, on a technical basis, be limited to professional microbiologists of the Agency. Ratification on policy, legal, and/or administrative grounds should be performed by appropriate personnel, not necessarily professional microbiologists.

FECAL COLIFORMS

Edwin E. Geldreich*

In 1904 Eijkman (1) discovered that coliform bacteria derived from the gut of warmblooded animals would produce gas from glucose broth at 46°C while the coliform strains from non-fecal sources failed to grow. Subsequent research into this concept by many investigators produced a variety of modifications in methodology which have resulted in increased sensitivity and selectivity. Major imporvements include the change to a modified lactose broth fermentation (2, 3); reduction in the elevated temperature requirement from 46° to 44.5°C; the use of primary enrichment for the multiple tube procedure (4, 5); and development of a routine (24-hour) membrane filter procedure (6), a rapid (7-hour) method for use either in emergency testing of water supplies or in quick appraisal of bathing water quality (7), and a delayed incubation technique where sample transit time exceeds 8 hours (8).

Standard Methods

Standard Methods (9) presents several procedures for detecting fecal coliforms in water. The fecal coliform test is easily accomplished by a multiple tube procedure (10) with minimal laboratory effort per analysis. The multiple tube procedure is the preferred method for the quantification of fecal coliforms when examining turbid waters or chlorinated primary effluents because of the difficulties of applying the membrane filter (MF) procedure to these waters.

The most common approach is to inoculate EC broth tubes from each Presumptive Test gas-positive tube using either a transfer loop or applicator stick. EC broth tubes are incubated at 44.5°C (± 0.2°C) for 24 hours and then examined for gas production. Positive-negative tube combinations can then be determined and the MPN calculated.

As an alternate procedure, each Presumptive Test gas-positive tube is confirmed in Boric Acid Lactose Broth and incubated at 43°C (± 0.2°C) for 48 hours. Data developed from testing both procedures in parallel on a wide variety of water sources, fecal specimens, and environmental samples indicate that Boric Acid Lactose Broth has essentially the same selectivity and sensitivity as the EC medium. However, the use of this medium does require 48 hours incubation to achieve results equivalent to the 24-hour confirmed EC test. Thus, EC broth is the medium of choice.

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*Water Supply Research Laboratory, NERC-Cincinnati

The direct inoculation of sample aliquots into EC tubes without preliminary enrichment in a presumptive test medium is unsatisfactory. Unpublished data from our laboratory show the direct application of the selective EC medium and immediate incubation at 44.5°C resulted in the detection of an average of only 24 percent of the coliform population in 88 fecal specimens obtained from human and farm animals. Parallel examination with the use of the recommended EC confirmation procedure resulted in 90 percent recovery of fecal coliforms from the same specimens.

The need for presumptive enrichment was also demonstrated in studies of the minimum Escherichia coli cell density necessary to produce gas in EC broth. The majority of 24 E. coli strains tested required from one to 20 viable cells to yield a gas positive reaction in EC broth, incubated for 24 hours at 44.5°C. However, three E. coli strains required 500 or more viable cells per inoculum, thus demonstrating that significant variability may occur. An optimal cell density, generally in excess of 1,000 viable organisms, is ensured when culture transfers are made from the Presumptive Test gas-positive tubes incubated at 35°C to the more selective EC broth incubated at the elevated temperature.

Standard Methods also includes a MF procedure for detecting fecal coliforms. In this procedure, appropriate sample volumes are filtered through the MF, then placed on an absorbent pad saturated with M-FC broth (6), and contained in tight fitting plastic Petri dishes. These cultures are then inserted in plastic bags and submerged in a water bath at 44.5°C (± 0.2°C) for 24 hours. Following incubation, the MF cultures are then examined under low-power magnification for fecal coliform colony occurrences; all blue colored colonies are then counted; and the fecal coliform density per 100 ml are calculated. Test accuracy has been found to be approximately 93 percent for a variety of samples.

Other Candidate Methods

The International Standards for Drinking Water (11) recognizes confirmatory fecal coliform tests utilizing a choice of brilliant green bile (BGLB) broth (12), formate-ricinoleate broth (13), or MacConkey broth (14, 15) in the multiple tube procedure. Fecal coliforms are considered to be present if gas production occurs with 6 to 24 hours incubation at 44°C (16). These confirmatory media offer no greater convenience over EC broth and somewhat lower sensitivity for the fecal coliform population. Preliminary studies comparing the use of EC and BGLB media in the elevated temperature confirmatory test (44.5°C) indicated that BGLB detected only 72.2 percent of the fecal coliforms in 24 hours whereas 92.1 percent were detected after 48 hours incubation when 24-hour EC results were used as the base line. Significantly lower recoveries can also be expected for lactose ricinoleate broth. In contrast, MacConkey broth used in the elevated temperature test has been reported to yield some false positive reactions because of growth of spore-forming anaerobic bacteria (14).

With respect to MF procedures, alternative methods have also been proposed. One technique employs an initial 2-hour enrichment on nutrient broth at 37°C followed by transferring the MF culture to a modified MacConkey broth for 16 hours incubation at 44°C (16). In another method, 0.4 percent Teepol broth is the sole test medium but cultures are initially incubated at 30°C for 4 hours and then at 44°C for a final 14-hour period. MF cultures from chlorinated samples are incubated at 25°C for 6 hours followed by incubation at 44°C for 18 hours (17). This more complicated incubation temperature scheduling can be accomplished either by transferring cultures from one incubator to another or by using a water bath that provides a programmed temperature change at the appropriate time.

The use of programmed media and temperature changes locks the laboratory into a very limited and rigid schedule that can severely restrict time for processing samples during working hours. Alternatives include extending laboratory hours, using two work shifts to cover the critical transfer time periods, or developing a dependable series of automatic water baths capable of programmed temperature changes.

Investigation of the modified MacConkey medium revealed an occasional difficulty with development of a consistent discrete yellow color confined to individual colonies. Heavy colony concentrations and long-term incubation may make accurate counts more difficult. Colony counts should be made promptly after removal from the incubator to reduce false positive occurrences.

Tryptose bile agar (TBA) medium combined with an indol test has also been used to detect fecal coliforms (18). In this method, the MF is placed on the TBA medium and incubated 20 to 24 hours at 44.5°C; then, the MF is transferred to an absorbent pad saturated with indol reagent. Indol positive colonies, E. coli Type I, become dark red upon reaction with this reagent. Such a procedure is not strictly a fecal coliform test since it detects only those fecal coliforms that are indol positive. In addition, verifying the results of this procedure has presented some difficulty because the indol reagent is toxic and prevents colony transfer. Discernment of reaction on adjacent colonies is also difficult because of wide zone of color development around indol positive colonies.

Rapid Methods

Rapid assessment of the sanitary quality of water is often needed for emergency or temporary potable water supplies, bathing beaches whose quality may have deteriorated following storms, and shellfish growing areas subject to sewage pollution. Some approaches to the quick determination of water quality have utilized C^{14}-labeled sodium formate in a rapid (4 hr) test for total coliforms (19, 20). The procedure shows considerable promise when used for fecal coliform detection but must be refined for greater reproducibility and increased sensitivity to coliforms at concentrations below 100 organisms per 100 ml. A membrane filter-fluorescent antibody (MF-FA) technique has also been proposed for rapid identification of fecal coliforms (21, 22). Before the MF-FA test for fecal coliforms can be considered practical, however, commercial polyvalent antisera must be developed that include all 145 "O" antigens and 86 "K(B)" antigens identified with the E. coli group (23) plus a few Enterobacter and Klebsiella strains also defined as fecal coliforms. To date, the three commercial polyvalent antisera contain only 20 "O" and "B" serotypes.

These rapid methods may not be adaptable to true emergency situations where skilled personnel and specialized equipment are not available. At present the most promising approach involves use of a new MF procedure utilizing a lightly buffered, lactose-based medium containing an acid-sensitive indicator system and incubation in a water bath at 41.5°C for 7 hours. Colonies should be examined at 20 to 30X magnification and all yellow colonies, both pale and bright, should be counted as fecal coliforms. In a study of colony verification, 94.3 percent of 4,082 yellow colonies from the 7-hour medium were verified as fecal coliforms and, from the same samples, 93.7 percent of 4,034 blue colonies on the M-FC medium after 24 hour incubation, were verified as fecal coliforms. These data indicated that both media are measuring essentially the same population of organisms. Advantages of this procedure include the 7-hour time factor versus the 24-hour normal test time and the knowledge that all necessary test equipment is normally available in most bacteriological laboratories.

Delayed Incubation Procedure

Sample transit time (8-hour limit) is especially critical for bacteriological examination of waters from stream pollution investigations or from the monitoring of remote sampling locations. Where special courier service or the use of mobile laboratory units is not feasible, the use of a delayed incubation test for fecal coliforms is the solution. In this procedure, MF cultures are held on vitamin-free casitone (VFC) holding medium during transport to the processing laboratory (8). Laboratory and field results have shown that inoculated MF can be held on VFC holding medium for up to 72 hours with little effect on fecal coliform counts. Upon arrival in the laboratory, the MF cultures are promptly transferred from the VFC holding medium to m-FC broth and incubated, submerged, in a 44.5°C water bath for 24 ± 2 hours. Following incubation, fecal coliform colonies are counted as in the standard fecal coliform test. Correlation coefficients between the delayed MF procedure and the immediate fecal coliform test for 1, 2 and 3 days were 0.93, 0.95 and 1.09, respectively.

Suggested Recommendations

Fecal coliform methodology must include both a multiple tube method and a MF procedure for flexibility in applications to field investigations and monitoring requirements. Where sample transit time is beyond an 8-hour limit, a suitable delayed MF procedure is needed as well as some simplified emergency procedure for rapid detection of fecal coliforms. Methods chosen should be reproducible, have a high order of specificity for the fecal coliform group, and utilize equipment and materials that are readily available.

To meet these criteria it is proposed that methodology for the multiple tube test be restricted to EC confirmation at 44.5°C for 24 hours after transfer of culture from gas positive 24-hour or 48-hour tubes of lauryl tryptose lactose broth incubated at 35°C. The recommended MF procedure should involve the use of M-FC broth, with incubation of cultures in a water bath at 44.5°C for 24 hours. These two tests are included in Standard Methods for the Examination of Water and Wastewater (9). For those special cases involving emergency testing of water supplies suspected of being contaminated and decisions on beach closings or openings, the 7-hour, rapid, FC test must be considered. Finally, a delayed procedure that employs VFC holding medium has proven practical and fills a definite need in the quantitation of fecal coliforms.

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FECAL COLIFORM DISCUSSION

Bordner: Will you make a comment on the possibility of applying the FC test on industrial effluents in light of Klebsiella pneumoniae occurrences?

Geldreich: At the Denver Conference we concluded that (1) Klebsiella pneumoniae is an inhabitant of the intestinal tract, (2) inclusion with the FC group as defined at 44.5°C is not detrimental to test interpretation, and (3) the presence of Klebsiella in the fecal material of over 40% of humans, indicates we can't ignore it.

Bordner: Well there was some talk about elevating the incubation temperature to 45.0°C.

Geldreich: Elevation of the test to 45.0° was proposed not so much to eliminate Klebsiella detection, but to screen out non-fecal coliform organisms that, at times, do cause interferences in fecal coliform colony discernment.

Berg: Do any of the Klebsiella that are growing out on M-FC medium actually appear as fecal coliforms?

Knittel: Yes, we have recovered several strains that give typical dark blue colonies on M-FC medium and they are EC+.

Stang: Will raising the temperature from 44.5 to 45.0°C cause some loss of fecal coliforms?

Geldreich: I see no problem in this change to 45.0°C. However, when incubation temperatures exceed 45.7°C, we are in trouble because of the sharp die off of fecal coliforms. We had originally set the temperature tolerance at ± 0.5°C. However, the shellfish program felt that this was too broad a tolerance. Thus, to be compatible, we agreed to make the tolerance ±0.2°C since there are many labs along the coast that examine both shellfish waters and fresh waters. Having the same temperature tolerance limits for both types of sample circumvents the need for two different water baths, differing by less than 0.5°C.

Cabelli: We have data from several different estuarine areas along the east coast where we have used M-FC medium. We get sometimes as low as 40% recovery using the MF procedure compared to another unselective method where the colonies are confirmed. So I would caution, in terms of estuarine waters at least, that the MF fecal coliform test may produce underestimates.

Geldreich: I would like to zero in a little closer on that problem by warning that before condemning a procedure, an investigation of the commercial media supply must be made. Don't comdemn a procedure until you are certain that the problem is not with a bad lot of commercial media. I have become so alarmed about the problem of commercial media quality that I am seriously considering preparing a questionnaire for the response of all EPA and State Laboratory Directors, as well as all interested bacteriologists in various federal, State, county, city, private, water treatment and sewage treatment laboratories, to determine what problems they have had with the quality of commercial media supplies. I have recently been in several State Health laboratories where I was informed of low recovery and poor colony differentiation with one brand of M-Endo medium. The manufacturer did exchange the bad lot for another lot of M-Endo medium but no effort was made to recall the defective lot form lab suppliers or unknowing consumers. Similar problems have been noted with lots of M-FC medium, BGLB broth, and standard plate count agar. What Bordner and his group must include in their activities is a media evaluation program or some mechanism to assist with the standardization of media, materials (MF) and instrumentation.

Cabelli: This problem is entirely possible. However, at least in our own studies, we used two different lots and two different manufacturers and ran into the same problem. It may be that this problem is widespread in estuarine waters, and I would, therefore, like to offer a word of caution when using this procedure with estuarine waters.

Geldreich: If this is true then we must use the multiple tube procedure in these instances.

Cabelli: Concerning Klebsiella and industrial wastes; we took a look at textile finishing plants where there is the same kind of problem as with paper mill effluents. In particular, there was one plant where there was no conceivable way they had fecal pollution. In fact, what they do is use sewage as a starter or fermenter to degrade the starch materials. There was never any feces in the "starter" and this fermenter has been in use for two years. There is no history of feces in this material.

Geldreich: Once you introduce this sewage "starter" they [fecal coliforms] will persist for long periods. We had this problem with sugar beet wastes.

Cabelli: The point is how do you justify that there is in fact fecal pollution? There is no history of feces being in this material.

Geldreich: If those organisms can survive in the "starter" for two years we are concerned that pathogenic organisms could also survive during this period.

Cabelli: Obviously there is no indication there are viruses present in this "starter," so now we must be talking about Salmonella.

Geldreich: -- or other pathogens, not necessarily just Salmonella, that are capable of persisting in that fermenter environment for long periods.

Knittel: [We have been doing studies] using a continuous flow system composed of primary settled pulp mill effluent inoculated with sewage. After three months, Klebsiella are beginning to take over. A profile of coliforms has been done. At the start Klebsiella occurred in less than one percent of the samples; now it occurs in more than 80 percent. As far as any significance to fecal pollution, [I feel] as long as they can grow other pathogens could also grow.

Cabelli: Can you define fecal pollution from a legalist point of view in this case?

Geldreich: Yes, it started with fecal pollution and we are working with something that has persisted in culture; now it is an entirely different menstruum.

Cabelli: Now you're talking about pathogens that are surviving on nutrient pollution, but can you say this is fecal pollution, per se?

Shedroff: I don't know the answer to that. What we are talking about in development of waste discharge permits is a limit that will indicate that there is no fecal pollution; that the material has been treated properly under the definition of best practicable treatment of the waste. It is not necessary to make that tie in with pathogens. It is only necessary to say that if you have properly treated you will have a certain level of quality and this is the quality we are expecting. You just don't have to demonstrate pathogens. However, the legislation being developed to define quality of industrial effluents may require a valid indicator to show that a particular effluent has been treated properly, as required.

Brezenski: Fecal pollution implies a hazard and I don't care if those organisms are from recent feces or have been in the system for some time. The point is that they are organisms that indicate a public health hazard.

Berg: The point is, these organisms were recovered from a supply of water of some sort and, in terms of a positive reaction at 44.5°C, are indicators of fecal pollution.

Are there other organisms that have been shown to grow at 44.5°C on M-FC medium which are not fecal coliforms?

Geldreich: There is a recent report in Applied Microbiology of finding one "odd-ball" organism in hot spring water from Yellowstone National Park. There is a possibility that some false positive organisms could grow on M-FC medium at 44.5°C, such as some thermophiles. The key is the requirement for lactose fermentation at the elevated temperature. This eliminates a good portion of the thermophilic population that could otherwise cause a problem.

Berg: I recall reading reports that Aeromonas will grow on M-FC medium and this organism is found in streams.

Geldreich: Aeromonas has been reported in this year's German literature to be capable of growth at an elevated temperature. These authors had suggested some modifications in their medium (not M-FC medium) to suppress Aeromonas, which was giving a problem of interference. I don't know what it would look like on the medium we developed. I have not had a chance to check out the growth response of Aeromonas on M-FC medium, and, therefore, am not aware of the colony color or possible problems of interference. Of all of the organisms we have studied on M-FC medium (over 10,000 strains), we have never identified an Aeromonas to date, so that is all I can say, but that is not all inclusive.

Berg: All I am trying to point out is that in a court of law this question could be brought up.

Geldreich: Aeromonas is becoming a real problem in fish hatcheries as a fish pathogen. There is some evidence of this problem in the recent literature. In discussions with Bobby Carrol, this morning, he expressed a concern about this organism as a fish pathogen in the Region IV area. Thus, although Aeromonas may not be related to a human health hazard, it may be a problem in a very unique way.

Carrol: The contact we have had with *Aeromonas* was primarily in a wildlife area. *Aeromonas liquifaciens* was a secondary invader causing haemorrhagic septicemia in fish. This was attributed to be the casue of a fish kill.

Cabelli: We have been looking at methods for *Aeromonas* detection in marine and fresh waters and have looked at *Aeromonas* occurrence in natural waters. In our studies we have not found *Aeromonas* growing on M-FC medium. We have found *Aeromonas* growing on M-Endo broth which presents a real problem. *Aeromonas* can be isolated in large numbers; 1 or 2 orders of magnitude less than total coliforms in raw and treated sewage.

Geldreich: *Aeromonas* has been reported in the current literature to occur in very high numbers when the total coliform population is very low. There may be, therefore, some difficulty in interpreting the sanitary significance of *Aeromonas*. Their occurrence is a problem in fish culture, but whether we can use this group as an indicator of human health hazard in bathing waters is debatable. There is beginning to appear a tremendous amount of literature indicating *Aeromonas* may be present in natural waters in high densities, but what is the significance?

Cabelli: I think I can address myself to that question. First of all, we have looked at *Aeromonas* in Narragansett Bay and as you go away from a pollution source the *Aeromonas* levels decrease. Near the pollution source *Aeromonas* densities are high. Secondly, it is capable of multiplying, at least in fresh water environment. Thirdly, *Aeromonas* is a pathogen, as can be cited in the literature, being isolated from urine, feces and even some systemic illnesses. We are speaking not of an indicator but a pathogen which has the ability to multiply in the aquatic environment. This being so, you can get higher counts in the stream than were shown to be discharged. It is in the feces of a certain number of individuals, and, from the work we did, it seems its erratic appearance in sewage suggests it is multiplying in the course of the treatment process. Furthermore, it can multiply in the aquatic environment, which means the numbers you find may be disproportionate to the numbers one would expect out of feces. Bonde, I think, has suggested we consider Aeromonas as a possible indicator. I would suggest we may have to worry about Aeromonas as a potential pathogen for humans as well as fish. The primary Aeromonas fish pathogenic strain will not grow at the elevated temperature. In fact, it does not grow well at 37°C unless you employ a very enriched medium.

Geldreich: In discussions with Dr. Bonde last March, in a WHO Conference on methodology for measuring bathing water quality in coastal areas, I was convinced he is not that strong about the use of Aeromonas. Dr. Bonde would prefer that the fecal coli test be used, which is identical to our definition of a fecal coliform test. That is, most European bacteriologists don't run out IMViC's on the gas positive results at the elevated temperature. He did say that he can find Aeromonas present in large populations in the estuarine environment, but does not know how to interpret their significance. This WHO Conference chose not to consider Aeromonas as a parameter for bathing water quality at this time because of the confusion as to their significance.

You know what this differnece really is between the E. coli test and a fecal coliform test--only a 5 to 7% difference. Thus, if we were to narrow the definition of fecal coliform to E. coli and exclude Klebsiella pneumoniae, we are going to be accepting a larger health risk. The British are actually using an enrichment procedure, then confirmation in a lactose type broth at 44°C; generally ending the test there. It is not always true that an indole test is being done in those laboratories as part of the confirmatory procedure. Much of their monitoring of water quality by the elevated temperature test is very similar to our fecal coliform test, but involves glutamic acid broth at 37°C, with confirmation in brilliant green broth or MacConkey broth at 44°C.

Cabelli: The IMViC test is not adequate and we should be looking at other biochemical tests such as the decarboxylase reaction. It is now time to go back and look at all of these kinds of environments and look at the composition of the coliform population; i.e., whether they are influenced by certain kinds of pollution; what is the *Klebsiella* portion of the population, what proportion of the population is *Aerobacter aerogenes*, or *Enterobacter cloacae* or *E. coli?*

Geldreich: This would be like discovering the wheel all over again--going back to repeat so much old research ground.

Cabelli: Well, you know environments change, societies change and the nature of pollution changes. Thus, those things that were true at one time may not be true now.

Resi: One of the things I want to bring up is the temperature requirement for the fecal coliform test. The use of ± 0.2° for temperature tolerance is difficult to obtain in the field. Ed seems to think this temperature tolerance is obtainable very readily, but I question that it is.

Berg: I can tell you there is no problem in maintaining 0.2°C in a system if you use a system capable of doing it. We do it all the time.

Resi: I agree with this but how many people do this all the time?

Geldreich: Critical factors in maintaining close temperature tolerance include gable tops over baths, some method to defeat temperature stratification, bi-metallic strips in the thermostatic control that are sensitive to the subtle changes in temperature and an accurate thermometer.

Gordon: Small commercial units are very good in the laboratory but you go out into a field laboratory where we don't have temperature controlability. With a small water bath that can be put in a plane you get stratification, and fluctuations in temperature become a problem.

Geldreich: Now we are talking about the real world of field laboratory work where these problems can be difficult to surmount.

Shedroff: Now we are talking about not what a research lab can obtain in temperature tolerance and other refinements but primarily what an industry laboratory or state laboratory can obtain on a regular basis.

ISOLATION AND IDENTIFICATION OF KLEBSIELLA PNEUMONIAE

M. D. Knittel, Ph.D.*

It has been considered for many years that coliform bacteria with an IMViC profile of --++ were of soil or plant origin and when found in water were of no sanitary significance. All of the tests of microbiological potability of water were arrived at to separate the fecal coliforms from the non-fecal coliforms. The IMViC determination represents the most used tool to accomplish this task.

Recent findings have shown that some waste water effluents contained high numbers of coliform bacteria with IMViC formulae of --++ (Bauer, unpublished results, EPA, Region X, Seattle, Washington). Further work demonstrated that these coliforms were non-motile and did not decarboxylate ornithine. These cultures were submitted to the U. S. Public Health Center for Disease Control and their finding was that these coliforms were *Klebsiella pneumoniae*.

It has been found (Knittel, unpublished data) that in spent sulfite liquor wastewaters, *K. pneumoniae* can constitute up to 90 percent of the coliforms present. At the same time, the elevated temperature and medium of the fecal coliform test will allow some *K. pneumoniae* to grow. Although most of these colonies are atypical, a few will appear as typical fecal coliform colonies, i.e., dark blue, flat colonies.

The significance of the occurence of *K. pneumoniae* in certain industrial effluents has been questioned by the National Council for Stream and Air Improvement(1). Whether *K. pneumoniae* is a primary health hazard or only an indicator of fecal pollution is immaterial; clearly, the presence of *K. pneumoniae* is itself an indication of conditions detrimental to human health.

The methods of isolation and enumeration of *K. pneumoniae* are not included in the recent edition of *Standard Methods of Water and Wastewater Analysis*. The methods used in that text are devoted to the selection and separation of fecal coliform bacteria. Therefore, any coliform bacterium that has an IMViC formula of --++ is discarded.

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*Pacific Northwest Environmental Research Laboratory, NERC-Corvallis.

Quantitative methods of isolation and enumeration of *K. pneumoniae* do not appear in the literature either. Most media that are inhibitory for coliforms are also inhibitory for *K. pneumoniae* and thus there is not a medium available for the direct enumeration of *K. pneumoniae*. The author has employed a basal salts lactose medium that was deficient in nitrogen. The lack of nitrogen was designed to take advantage of the ability of *K. pneumoniae* to fix atmospheric nitrogen(2). Preliminary evaluation failed to support the concept and further work was discontinued.

The following procedure is used at this laboratory to isolate and identify *K. pneumoniae* from samples of water and wastewater: Appropriate dilutions of the sample are filtered through a 0.45 Millipore membrane filter. After washing, the membrane is placed in a petri dish containing M. Endo LES agar (Difco, Detroit, Michigan) and incubated at 35°C for 24 hours. At the end of the incubation period a count of the sheen producing colonies is made and recorded.

A representative number of these colonies are picked at random and transferred to triple sugar iron agar slants (TSIA) and nutrient agar slants (NA).

These slants are incubated at 35°C for 24 hours. The reaction of the TSIA are recorded and indophenol oxidase test is performed on the nutrient agar slant (3). The TSIA slants that are indophenol oxidase negative and show a TSIA reaction of: acid slant and acid butt with or without gas and no H_2S production are considered to be coliforms. Transfers are made into tryptone, buffered glucose, citrate, malonate, motility, urea agar, KCN, Lysine, ornithine decarbonylase and arginine dihydrolase media. The above cultures are incubated up to five days at 35°C with daily observations.

Cultures isolated from a sample which are IMViC -(+) - ++, non-motile, lysine decarboxylase positive but arginine dihydrolase negative and ornithine decarboxylase negative, which are urea; KCN and malonate positive are *K. pneumoniae*. Further confirmation can be done by passing the culture in a medium to enhance capsule production such that of Hoogerheide (4) or Worfel and Furgeson (5) and then typing the K or capsular antigen.

The estimation of the percentage of *K. pneumoniae* can be calculated by dividing the number of *K. pneumoniae* identified by the number of isolates originally picked. This percentage times the total coliform count will give the number of *K. pneumoniae* in the original sample. Of course this is a relative and somewhat arbitrary figure. However, *in leu* of a selective medium for *K. pneumoniae* this procedure, at least, will give a first approximation of numbers of *K. pneumoniae* present in samples tested.

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DISCUSSION: A SUMMARY

The selection of colonies from the primary culture for identification of *K. pneumoniae* is necessary because of the lack of a selective or a differential medium for the bacterium. This approach does allow one to determine the number of *K. pneumoniae* in the original sample by calculating the percentage of the isolates that it represents. In addition, it also provides information as to what other types of coliforms make up the total population. This information could be useful in determining the source or fate of coliforms in water.

The classification of coliforms has undergone some changes from what is listed in *Bergy's Manual of Determinitive Bacteriology*. The separation of *K. pneumoniae* from *Enterobacter aerogenes* (*Aerobacter aerogenes*) was not certain and was based primarily on source, encapsulation and motility. Thus, a gram negative rod-shaped bacterium, that fermented lactose and was non-motile isolated from soil, plants or water was classified as *E. aerogenes* (*A. aerogenes*).

The development of a medium and procedures for determining the ability of bacteria to decarboxylate amino acids (Moeller 1965)* has provided a means to separate *K. pneumoniae* from *E. aerogenes* (*A. aerogenes*). This development has shown that not all of the IMViC --++ coliform bacteria are *E. aerogenes* (*A. aerogenes*). The presence of *K. pneumoniae* in certain industrial wastewaters raises the question whether these organisms are primary pathogens or indicators of fecal pollution.

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* Moeller, V. 1955. Simplified Tests for Some Amino Acid Decarboxylases and for the Arginine Dihydrolase System. Acta Path. et Microbiol. Scandin. 36, 158-172.

FECAL STREPTOCOCCI

Francis T. Brezenski*

In the past it has been the tendency to think in terms of a universal indicator system - one system which would apply to the highest percentage of cases or conditions that exist. It is apparent that as technology has increased, so has the diversity of waste materials. Problems with industrial wastes have arisen which indicate that bacterial parameters will have to be more specialized and show the closest compatibility or relevancy to the substrate being tested. Simplicity can no longer be a major or decisive consideration in choosing testing procedures. The most accurate, descriptive and rapid system is desirable. It is from this frame of reference that pertinent methodology is being chosen.

For a number of years the fecal streptococci have been one of the most controversial groups of bacteria. Microbiologists have had great difficulty in defining, utilizing, and interpreting data collected from fecal streptococcus assays. It is true that the fecal streptococci possess desirable characteristics which warrant consideration and further development. These are: host specificity, lack of multiplication in the aqueous medium, serological characteristics which demonstrate potential application to automated detection and identification systems and finally, an increased interest in the association of the Group D Streptococci in specific disease processes. On the other hand, many problems exist such as lack of standardization in biochemical testing. This is crucial since present identification is primarily based on biochemical characterization.

Standard Methods for the Examination of Water and Wastewater (SM), 13th Edition, 1971 lists three methods for the assay of fecal streptococci: Multiple Tube Technic (MPN), Membrane Filter Technic (MF) and a tentative Fecal Streptococcal Plate Count procedure. The MPN procedure involves a Presumptive Test using Azide-dextrose broth and a Confirmed Test utilizing Ethyl violet azide broth. The MPN test was developed to determine the density of enterococci in sewage. Density determinations are estimates based on statistical probability measurements. The more rapid MF technique, unlike the MPN procedure, is a direct measurement of fecal streptococci on a membrane filter. The test can be used to assay fecal streptococci in water and non-chlorinated sewage. A choice of either M-Enterococcus or KF agar medium is offered to the analyst. The Fecal Streptococcal Plate Count (FSPC) is a pour plate technique using M-Enterococcus or KF Streptococcus agar. It is suggested as an alternate method for the MF procedure when highly turbid waters containing low numbers of fecal streptococci are encountered. At the present time it is listed as a tentative procedure.

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*Technical Support Branch, Region II

In order to choose the most effective and expedient methodology, one must first define what the detection system is and what it is detecting. Secondly, one must know the sensitivity and reproducibility of the method. The first - definition of the detection system - needs to be made clear and agreed upon. To understand this, one must look at the major developments and present status of the fecal streptococcus group.

The fecal streptococci were proposed as indicators of fecal contamination as early as 1910. However, the United States application of this group of bacteria to pollution detection did not take place until the post World War II period. Since that time numerous reports have appeared in the literature concerning methods and media for the isolation and enumeration of the fecal streptococci. Sixty-eight (68) different types of media have been proposed in one form or another for the selective isolation of these organisms (1). Such a list demonstrates the difficulty and complexity in selecting out the microbes from the existing heterogeneous microflora. The diversity of this group of organisms created serious problems with taxonomy and classification. To further complicate the problem, a number of terms - (some used synonymously) appeared in the literature. Fecal streptococci, enterococci, Lancefield Group D streptococci, Enterococcus Group etc. were used to denote the bacteria in question. Numerous divisions in groups and species, involving biochemical reactions or serological analysis and/or other characteristics have been proposed.

The initial task therefore was to establish which streptococci are significant in determining the sanitary quality of the substrate being tested. Sherman (2) divided the streptococci into four basic groups: Pyogenic, Viridans, Lactic, and Enterococcus. This division represented a practical application to pollution investigation. The Viridans and Enterococcus Groups contained species which were associated with the alimentary tract of warm-blooded animals. Organisms from both groups were combined to form the Fecal Streptococcus Group. Hartman et al. (3) in 1966 defined the various species of the Fecal Streptococcus Group. The Enterococcus Group consisted of *S. faecalis* and its varieties *liquefaciens* and *zymogenes*, *S. faecium* and its variety *durans*. These organisms shared a common antigen, hence were serologically classified as Group D organisms. The Viridans Group organisms consisted of *S. bovis*, *S. equinus*, *S. mitis*, and *S. salivarius*.

The most important bond of the fecal streptococci was the fact that all of the species were associated with fecal material. It was not long, however, when Mundt (4) found that Enterococcus Group organisms may be chance contaminants of plants. Such organisms were recovered from a number of different plants in areas remote from man. This finding seriously challenged the group as strict indicators of fecal contamination.

A later report by Geldreich and co-workers (5) indicated that the presence of these organisms on vegetation may, at least in part, have been derived from insect contact. This seemed plausible since previous reports in the literature demonstrated the presence of Enterococcus Group organisms in various species of insects.

Besides establishing the levels and streptococcal types on vegetation and insects, Geldreich and co-workers performed concurrent coliform and fecal coliform determinations. Results suggested that typical fecal coliforms, as measured by the fecal coliform test, contributed only a small percentage of the organisms associated with insects and vegetation. Therefore, it was concluded that the contribution from warm-blooded animal pollution was a minor factor in both vegetation and dissemination by insects.

The higher densities of streptococci found on vegetation were explained as being streptococcal types not of warm-blooded origin. These strains hydrolyzed starch, even though all other biochemical criteria were typical for the Enterococcus Group as defined by Sherman (2). Strains of *S. faecalis*, however, isolated from water, warm-blooded animals, and cold-blooded animals have never displayed this characteristic. The starch hydrolyzing strains were called atypical *S. faecalis* and were interpreted as being of vegetative source.

A very recent report by Martin and Mundt (6) showed high numbers of Enterococcus Group organisms in insects collected from non-urban, wild and cultivated fields and woods. More than 93% of the cultures of S. faecalis isolated digest casein milk from the top downward, following the production of soft flowing curd. This property is not a characteristic of S. faecalis isolated from humans. The litmus milk reaction therefore discriminates between cultures associated with insects and vegetation and cultures derived from human origin.

The above has served to illustrate the need to specify which organisms form the basis of the detection system and how they can be discriminated from other forms. Discrimination was based on biochemical characterization. The techniques used for biochemical characterization are long, involved, quite vulnerable to error during laboratory manipulations and lack standardization. For these reasons, and the fact that the fecal streptococci share a common antigen, fluorescent antibody techniques have been proposed for the detection and identification of species belonging to the group.

In order to develop fluorescent antibody (FA) techniques for the identification of Group D streptococci, it was first necessary to isolate strains from a number of sources. Strains would then be used in the preparation of the conjugate which will form the basis of the detection system. The University of Massachusetts (7), in concert with the EPA Region II laboratory at Edison, New Jersey isolated a number of fecal streptococcal strains from various sources and developed a conjugate for the identification of Group D Streptococci. In order to ensure isolation of all fecal streptococcal strains present, five (5) different media containing different classes of inhibitory agents were used: M-Enterococcus, KF-Streptococcus, Thallous-Acetate, Azide-Sorbitol, and Pfizer Selective Enterococcus (PSE) Agars. KF and M-Enterococcus agar contain sodium azide and 2,3,5 triphenyltetrazolium chloride (TTC), while PSE incorporates bile and sodium azide as main constituents to attain selectivity. Domestic sewage and the feces of sheep, cattle, horses, rabbits, chickens, geese, and rats were analyzed for streptococcal species. Additional sources that were investigated included frozen and non-frozen foods.

The lowest level of fecal streptococcus recovery occurred on M-Enterococcus and Azide-Sorbitol Agars. Thallous-Acetate agar was the most productive, however, it was the least selective since 58% of the isolates were non-fecal streptococci. KF and PSE Agars yielded the highest recovery of fecal streptococci while exhibiting the lowest percent of non-fecal streptococci. PSE Agar recovered a higher percent of non-fecal streptococcal types (23%) than did KF (19%). The advantage of PSE is that it only requires half the incubation time required by KF. As a result, fecal streptococcal counts are available after 24-hours incubation. In summary, PSE exhibited a consistently higher recovery from a wide range of sources which included foods, animal feces, and domestic sewage. The medium allowed the growth of all types of fecal streptococci while exhibiting a slightly higher percent of non-fecal streptococci than did KF. Results from PSE plates are available after 18 to 24-hours incubation.

The fresh isolates obtained from sewage, feces of sheep, cattle, horses, rabbits, chickens, geese, and rats were used to develop FA techniques for the identification of Group D Streptococci. The species isolated were initially identified by conventional, physiological, biochemical, and serological tests. Both whole and disrupted cells of representative strains of each species were used for the preparation of the Group D Streptococcus vaccine. Globulin fractions of individual and pooled antisera were labeled with fluorescein isothiocyanate dye (FITC), and the resulting conjugates were tested with homologous and heterologous antigens. Conjugate specificity was determined by adsorption and inhibition tests using controls with homologous and heterologous antigens. Using the direct staining method and individual and pooled conjugates, it was possible to obtain 84 and 85% positive FA reactions, respectively, with Group D Streptococcal strains. Non-group D Streptococci and Staphylococci (FA cross-reactants) were eliminated by trypsinization of the smears prior to staining.

The indirect FA staining technique was also evaluated for use with the Group D conjugates used above in the direct staining procedure. Results indicate that the indirect method is more sensitive than the direct in its ability to identify the Group D Streptococci. However, the number of false positives due to cross reactants increases tremendously. Trypsinization did not remove the cross reactions. The more sensitive indirect technique had to be sacrificed in favor of the more specific direct method.

At the present time, the FA system for Group D Streptococcal identification is ready for field testing. Reagent standardization, optimum conditions for preparation of globulin to be labeled, the determination of the most optimum labeling conditions, the determination of optimum storage conditions of the conjugates and the application of photometric techniques for direct fluorescent measurements of specimens have been initiated. The data at the present time indicate that the FA method, using a direct staining technique, will be of value in the rapid identification of Group D Streptococci.

Summary

On the basis of the above information and experience gained to date, the following recommendations are being made:

1. The MF technique, using KF agar, be used to assay fecal streptococci in water samples and non-chlorinated sewage. The pour plate technique is recommended as an alternate procedure for the MF technic when chlorinated sewage effluent and water samples with high turbidity are encountered. PSE and KF agars are recommended as the pour plate media.

2. High recoveries of fecal streptococci from human and animal feces, a shorter incubation period (24 hrs.) and a low percentage recovery of non-fecal streptococci cause considerable interest in the PSE medium. Unlike the PSE pour plates, poor colony distinction occurs on PSE membrane filters. Efforts are presently underway to solve this discrepancy.

3. The identification of fecal streptococcal strains provide valuable data in determining the source of contamination. Biochemical tests used to achieve strain identification lack standardization and are the chief sources of error in strain identification.

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THE USE AND ABUSE OF FECAL STREPTOCOCCI IN WATER QUALITY MEASUREMENTS*

Edwin E. Geldreich**

The fecal streptococcus group does merit consideration in water quality criteria since it is now being recommended and used in many stream pollution measurements. Generally, the occurrence of fecal streptococci in water suggests fecal pollution and their absence indicates little or no warm-blooded animal contamination (1). Although fecal streptococci may persist for extended periods in irrigation waters of high electrolyte content, they rarely multiply in polluted water.

This group encompasses a wide spectrum of strains that have diverse survival rates and specific fecal origins and also includes several biotypes that are of limited sanitary significance (1-3). The ubiquitous S. faecalis var. liquifaciens (Figure 1) may frequently represent a substantial portion of any fecal streptococcus population in natural waters of good quality. These waters (Table 1) may be devoid of recent fecal pollution, receive only small additions of contamination, or contain minute vestiges of some pollutional discharge remote in time or place. Until better methodology is available to selectively exclude such strains of limited sanitary significance, the use of fecal streptococcus limits in recreational waters must be pegged to some maximum density above 100 organisms per 100 ml (4). An alternate approach for bathing waters would be to confirm the validity of very low densities of fecal streptococci by parallel examination for fecal coliform bacteria before making a decision to temporarily restrict use of that water.

In contrast, analysis of data on the distribution of subgroups of fecal streptococci in warm-blooded animal discharges reveal that S. bovis and S. equinus are specific indicators of non-human warm-blodded animal pollution (Figure 2). This is a particularly useful differential characteristic in pollution investigations involving cattle feedlot runoff, farm land drainage, and discharges from meat and duck processing operations, and dairy plant wastes. In addition, S. bovis and S. equinus are the indicator organisms showing the most rapid die-off outside the animal intestinal tract (Figure 3). Therefore the detection of these two strains in water indicates very recent farm animal contamination.

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* This paper submitted in writing for the record.

** Water Supply Research Laboratory, NERC-Cincinnati.

OCCURRENCE OF *S. faecalis* VAR. *liquifaciens* IN VARIOUS ENVIRONMENTAL SOURCES

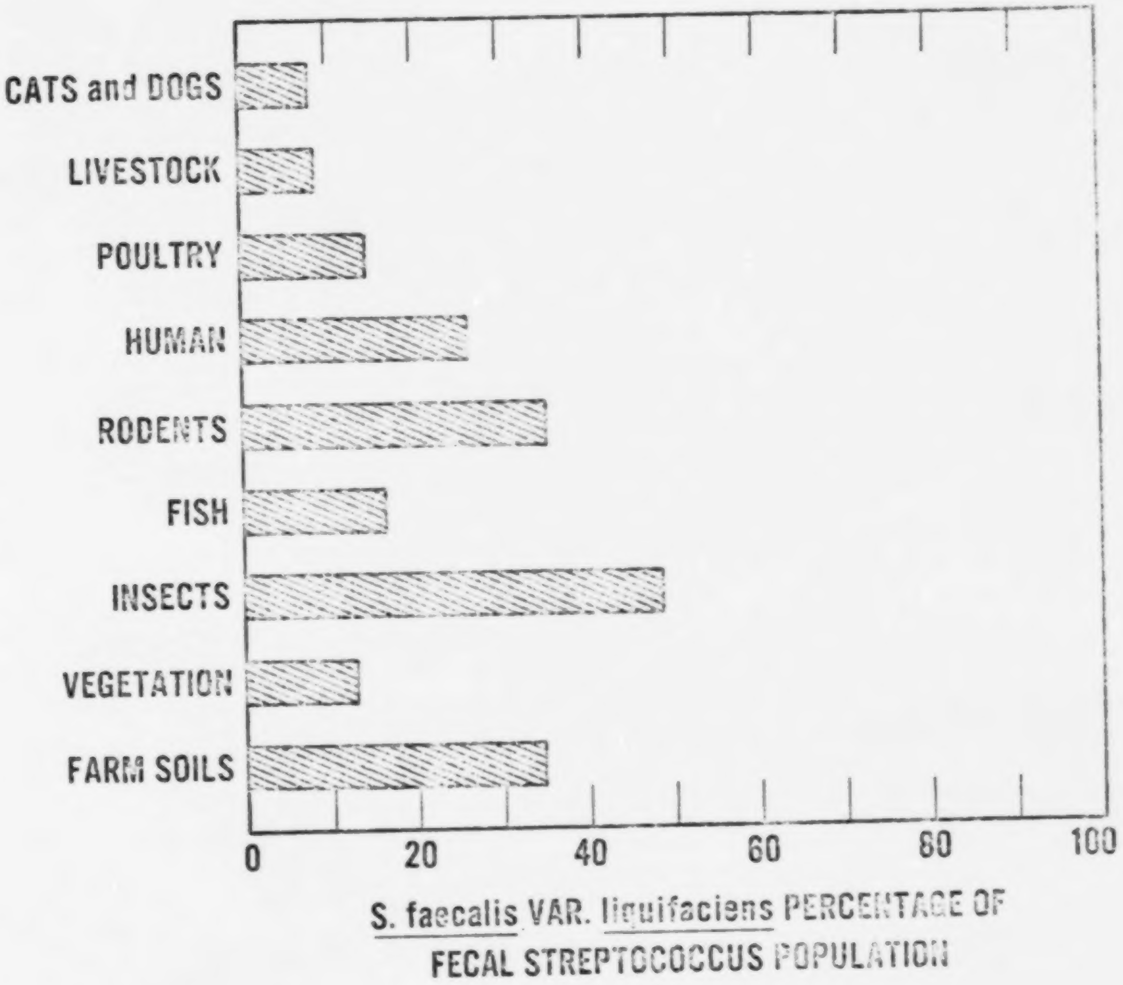
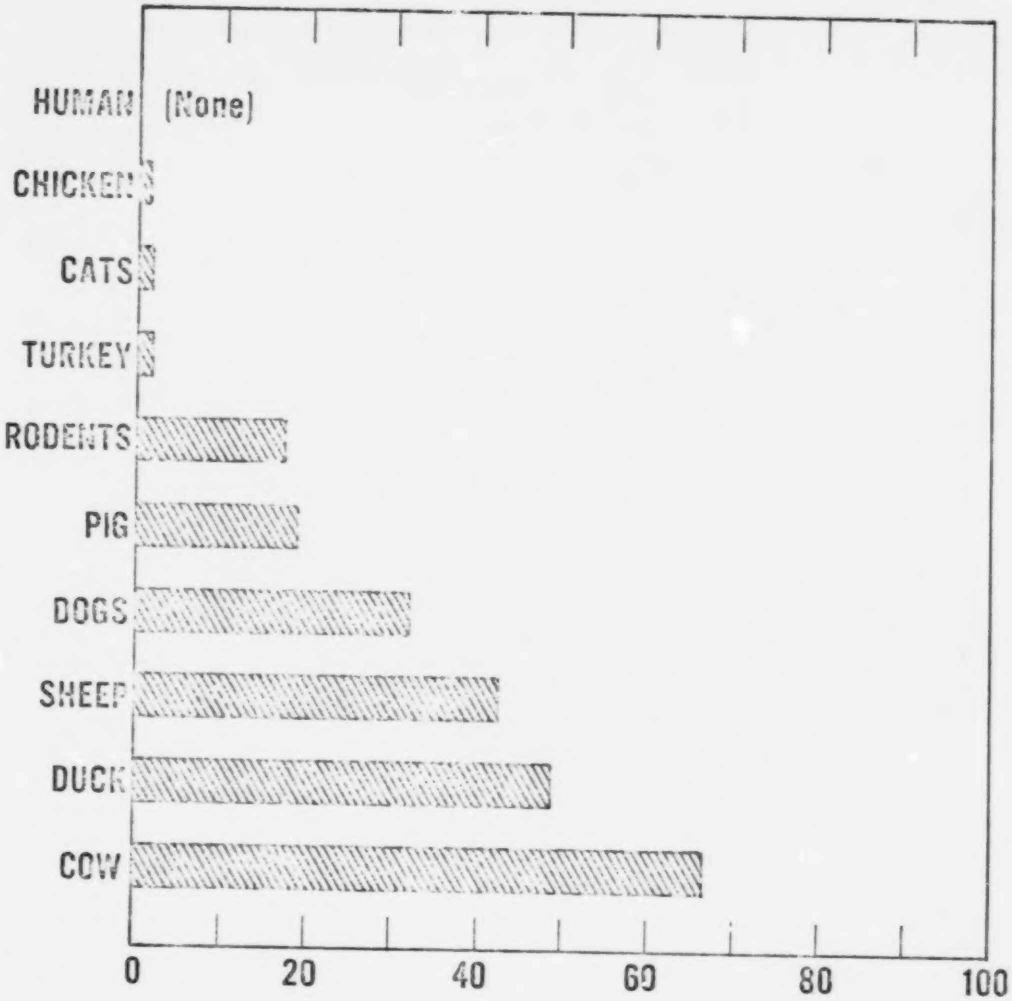


Figure 1.



S. BOVIS - S. EQUINUS PERCENTAGE OF FECAL STREPTOCOCCUS POPULATION IN WARM-BLOODED ANIMAL FECES

FIGURE 2.

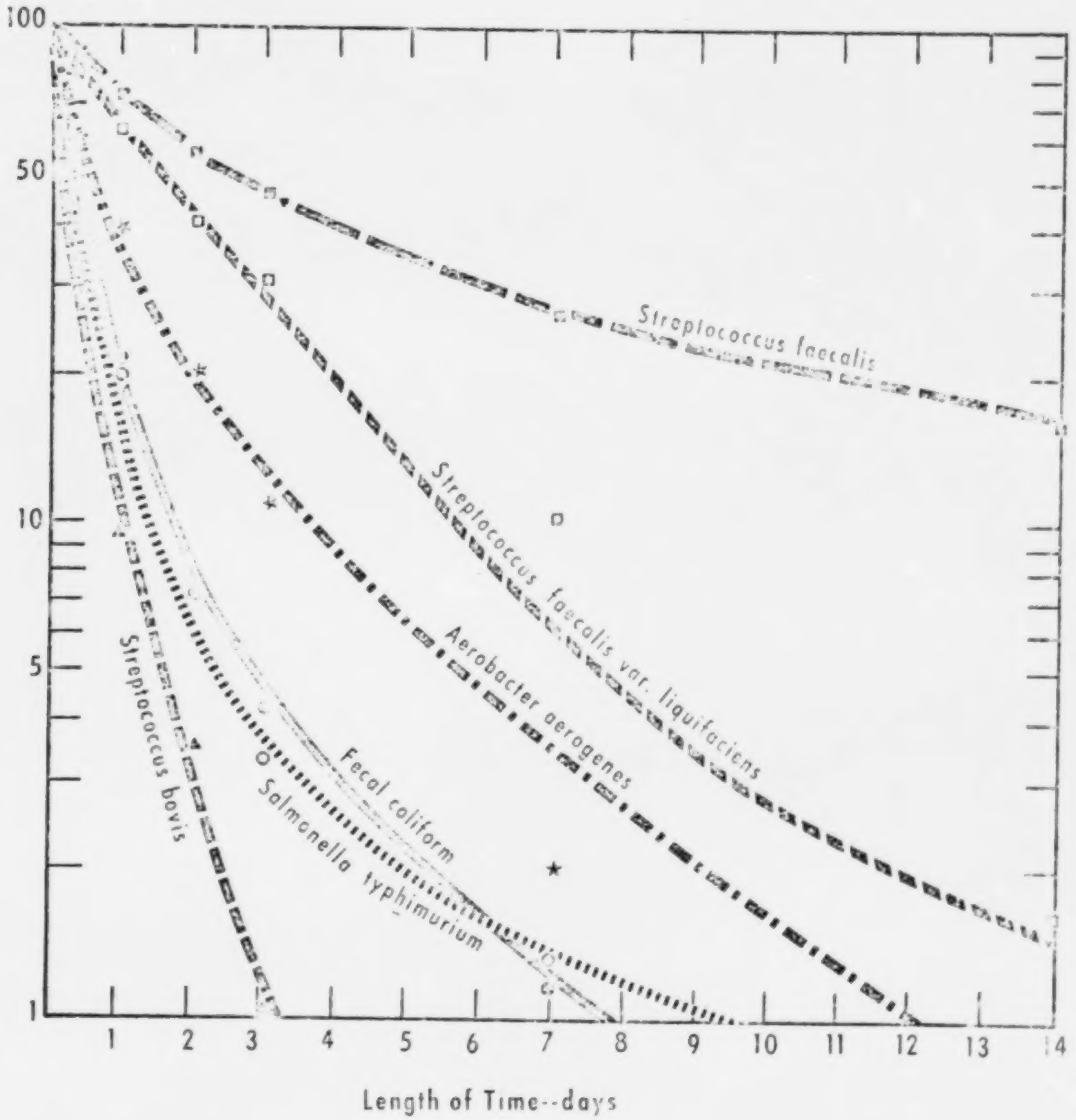


Fig. 3. Persistence of Selected Enteric Bacteria in Storm Water Stored at 20°C

TABLE 1. DISTRIBUTION OF _S. FAECALIS LIQUIFACIENS_ IN PRAIRIE WATERSHEDS, RECREATIONAL WATERS AND PRIVATE WELL SUPPLIES

WATER SOURCE	DENSITIES PER 100 ML*		Percent Occurrence
	FECAL COLIFORMS	FECAL STREPTOCOCCI	_S. FAECALIS LIQUIFACIENS_
PRAIRIE WATERSHEDS			
Cherry Creek, Wyo.	90	83	35.3
Saline River, Kan.	95	180	24.3
Cub River, Idaho	110	160	NONE
Clear Creek, Colo.	170	110	4.3
RECREATIONAL WATERS			
Lake Mead	2	444	4.0
Lake Moovalaya	9	170	22.0
Colorado River	4	256	NONE
Whitman River	32	88	NONE
Merrimack River	100	96	4.8
PRIVATE WELL SUPPLIES			
Gilman	0.8	8,800	100.0
Miami	1.0	18	NONE
Baas	1.1	32	26.7
Kilb	2.8	106	26.5
Hopper	5.0	134	NONE

*All bacterial densities for prairie watersheds and recreational waters were based on median values. Data for private well supplies were developed from single samples only.

TABLE 2.

FECAL COLIFORM TO FECAL STREPTOCOCCUS RELATIONSHIPS IN DOMESTIC SEWAGES AND HUMAN FECES

SOURCE	DENSITIES PER 100 ML EFFLUENT OR 1 GM FECES*		RATIO FC/FS
	FECAL COLIFORMS	FECAL STREPTOCOCCI	
DOMESTIC SEWAGE			
PRESTON, IDAHO	340,000	64,000	5.3
FARGO, N. D.	1,300,000	290,000	4.5
MOOREHEAD, MINN.	1,600,000	330,000	4.9
CINCINNATI, OHIO	10,900,000	2,470,000	4.4
LAWRENCE, MASS.	17,900,000	4,500,000	4.0
MONROE, MICH.	19,200,000	700,000	27.9
DENVER, COL.	49,000,000	2,900,000	16.9
HUMAN FECES			
43 SAMPLES	13,000,000	3,000,000	4.4

*MEDIAN VALUES

Another valuable application of the fecal streptococcus indicator system in stream pollution investigations has been through correlation with the fecal coliform group. Fecal coliform bacteria are more numerous than fecal streptococci in domestic sewages, with a fecal coliform to fecal streptococcus ratio always greater than 4.0(Table 3). As would be expected, similar ratios are common to the feces of man. Conversely, fecal streptococci are more numerous than fecal coliforms in the feces of farm animals, cats dogs, and rodents (Table 4). In feces from these animals, the fecal coliform to fecal streptococcus ratios are less than 0.7. Similar low ratios are common to urban stormwater and farmland drainage.

These fecal coliform to fecal streptococcus ratios must be applied carefully. Correlations are most meaningful when developed from bacterial densities in water samples taken at waste outfalls into a stream. Once these organisms are diffused into the receiving stream, various ecological forces may alter the interrelationship between these indicator systems during flowtime downstream. Under these conditions, the distribution of subgroups of fecal streptococcus strains within this pollution indicator group can be drastically altered by dilution with organisms in the receiving water or through selective adaption to the water environment by only a few vigorous strains. For these reasons, ratios for stream samples will be valid only during the initial 24-hour travel downstream from point of pollution discharge into the receiving river.

Areas of Research Development

In contrast to the milk and food environment, water is a severe menstruum for the survival and subsequent detection of bacterial indicator systems and any associated pathogens. Until recent years, fecal streptococci were considered by early investigators to be present in stream water in relatively low magnitude approaching a density of one tenth that of the total coliform population and always dying rapidly outside the intestinal tract (5-7). More recent media developments (8-11) have shown that the fecal streptococcus densities in polluted water approach the magnitude of the coliform population or at times exceed it by a factor of 10, depending upon the source of fecal pollution. Fecal streptococci are now observed to persist for long periods in stormwater and irrigation water containing large concen-

TABLE 3.

FECAL COLIFORM TO FECAL STREPTOCOCCUS RELATIONSHIPS IN STORMWATER RUNOFF, ANIMAL PETS, RODENTS, AND FARM ANIMALS

	DENSITIES PER 100 ML RUNOFF OR 1 GM FECES*		
SOURCE	FECAL COLIFORMS	FECAL STREPTOCOCCI	RATIO FC/FS
STORMWATER RUNOFF			
BUSINESS DISTRICT	13,000	51,000	0.26
RESIDENTIAL	6,500	150,000	0.04
RURAL	2,700	58,000	0.05
ANIMAL PETS			
CAT	7,900,000	27,000,000	0.3
DOG	23,000,000	980,000,000	0.02
RODENTS	160,000	4,600,000	0.04
LIVESTOCK			
COW	230,000	1,300,000	0.2
PIG	3,300,000	84,000,000	0.04
SHEEP	16,000,000	38,000,000	0.4
POULTRY			
DUCK	33,000,000	54,000,000	0.6
CHICKEN	1,300,000	3,400,000	0.4
TURKEY	290,000	2,800,000	0.1

*MEDIAN VALUES

TABLE 4.

FECAL STREPTOCOCCUS MEDIA EVALUATION ON OHIO RIVER WATERS*

RIVER STATION FEBRUARY 1970	DENSITIES PER 100 ml STREAM SAMPLE		
	FECAL COLIFORM	FECAL STREPTOCOCCI	
		KF AGAR	M-ENTEROCOCCUS
OHIO RIVER			
Mile 640	800	2,700	1,000
Mile 626	1,100	2,300	660
Mile 502	1,100	13,000	1,300
Mile 491	3,200	3,900	1,100
Mile 450	800	1,700	660
Mile 421	1,700	1,500	780
LICKING RIVER			
12th St. Bridge	1,400	4,800	1,300
LITTLE MIAMI RIVER			
Beechmont Bridge	6,800	22,000	6,100
GREAT MIAMI RIVER			
Seller Road	6,400	17,000	5,800
Liberty-Fairfield	11,000	17,000	4,700
American Mat.	9,200	9,000	6,800
Lost Bridge	9,000	14,000	4,800
WHITEWATER RIVER			
River Mouth	120	1,600	720

*Data courtesy of the Analytical Quality Control Laboratory, EPA trations of electrolyte and a favorable water temperature (1). Mundt et al. (12) have found some evidence of S. faecalis multiplication in waste waters of vegetable processing plants.

These increases in media sensitivity have not come about without some compromise in suppression of other organisms present in the water environment. Strains of Corynebacterium, Pediocossus, Streptococcus lactis and other organisms common to irrigation water, sugar beet plant effluents and other food processing wastes can develop substantial growth in either the liquid media or selective agars now available (12-15).

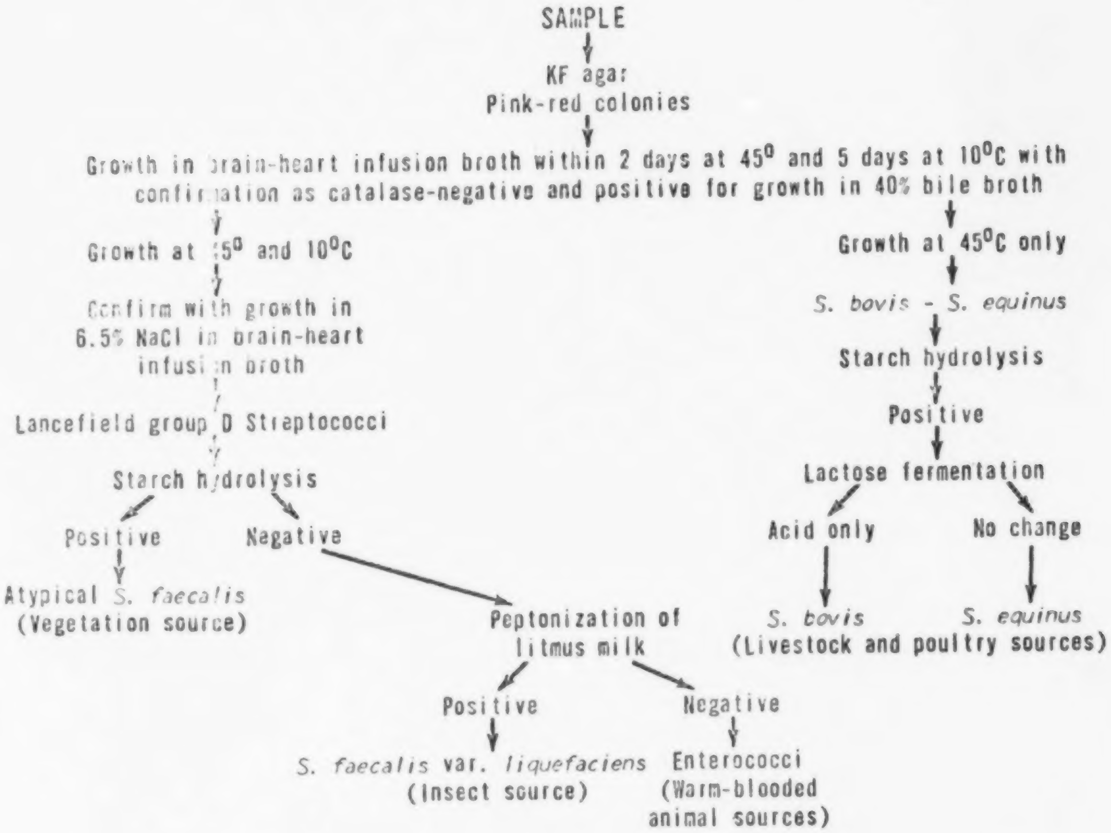
Maximum suppression of interfering organisms can, in part, be achieved through careful media preparation. Sodium azide, a frequent selective inhibitor in these media, does deteriorate with age in dehydrated media held for two years or more prior to reconstitution. Sterilization in autoclaves with long time cycles or contact with a chemically contaminated boiler steam source can cause a decomposition of sodium azide to form toxic acid products. Addition of a sodium carbonate buffer is sometimes necessary to prevent undesirable shifts in pH during sterilization. Poor differential colony color on both KF and M-Enterococcus agars can result from either an unsatisfactory grade of tetrazolium indicator or from its exposure to excessive sterilization temperatures. Laboratory experience indicated that a five minute exposure in a boiling water bath after the agar has melted, and addition of filter-sterilized tetrazolium after cooling will eliminate much of the irregularities observed.

Lack of uniformity in media sensitivities has been observed during comparative studies using stream samples. Data presented in Table 5, which were supplied by courtesy of the Analytical Quality Control Laboratory, FWPCA, revealed significant differences in the fecal streptococcus densities detected by M-Enterococcus and KF agars. The question of which medium is more accurately detecting the fecal streptococcus density along a 220 mile reach of the Ohio River and its tributary streams can only be resolved through verification of the colonies by selected biochemical tests. Further colony confirmation would indicate whether KF medium needed to be incubate at 44.5°C to suppress non-fecal streptococcus organisms or whether M-Enterococcus agar needed prior enrichment to increase recovery of all strains of fecal streptococci. Other comparative studies frequently have shown both media to yield essentially equivalent results on sewage effluents. However, KF agar recoveries were higher on samples examined from cattle feedlots, meat packing house wastes, and farm drainage because KF agar apparently gave better recovery of the *S. bovis* and *S. equinus* strains.

Normally there is no need for species identification of fecal streptococci in stream pollution studies. Density relationships with fecal coliforms are adequate to assign the probable source of waste discharge as being domestic or from farm animals and wild life. However, special applications involving: tracer organism identification, confirmation of sanitary significance of very low fecal streptococcus densities, and media evaluations will require further biochemical identification (1, 19-21). Practical application of identification procedures demands a simplification of the tests and more specific biochemical reactions. Further development of a serological schema, which currently includes 39 serotypes, could be an important breakthrough in this problem (21, 22-27).

SUMMATION

The true sanitary significance of fecal streptococci has been confused somewhat by controversies concerning procedures for quantitation, definition of the group, and differing concepts as to their occurrence in the water environment. It is true that there are problems in methodology yet to be resolved which include: improvements in media formulations, possible application of elevated temperature incubation, and simplification of biochemical tests or development of a serological procedure for use in species or subgroup identification. However, recognition of these areas of future research should not detract from use of the fecal streptococcus group in stream pollution measurements.


SAMPLE
KF agar
Pink-red colonies
Growth in brain-heart infusion broth within 2 days at 45° and 5 days at 10°C with confirmation as catalase-negative and positive for growth in 40% bile broth
Growth at 45° and 10°C
Confirm with growth in 6.5% NaCl in brain-heart infusion broth
Lancefield group D Streptococci
Starch hydrolysis
Positive
Negative
Atypical S. faecalis (Vegetation source)
Peptonization of litmus milk
Positive
Negative
S. faecalis var. liquefaciens (Insect source)
Enterococci (Warm-blooded animal sources)
Growth at 45°C only
S. bovis - S. equinus
Starch hydrolysis
Positive
Lactose fermentation
Acid only
No change
S. bovis
S. equinus
(Livestock and poultry sources)

Note: A small percentage of unclassified strains (biotypes) will be found since fecal streptococci vary greatly in their biochemical reactions.

Figure 1. Schematic outline for identification of fecal streptococci.

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FECAL STREPTOCOCCI - DISCUSSION

The membrane filter method or agar pour plate technique are recommended over the multiple tube procedure (MPN) for the enumeration of fecal streptococci because:

(a) Recoveries on current MF media are higher and are less affected by interference organisms.

(b) A higher number of false positive reactions occur in broth MPN systems.

(c) When group/or species identification are required, it is necessary that preliminary isolation be made on solid media. MF and agar pour plates readily allow for primary isolation of fecal streptococcus colonies.

However, certain environmental conditions prevent the use of the membrane filter technique, e.g., waters with high turbidity and waters with high concentrations of heavy metals or other toxic compounds. Under those conditions, the AD-EVA multiple tube technic is recommended. (Azide - Dextrose - Presumptive and Ethyl Violet Azide - Confirmatory Tests.) It must be realized that a high number of false positives may occur, especially in seawater, and streaking of a percentage of the positive tubes on agar medium for subsequent colony verification is mandatory.

Data on fecal streptococcus recovery from animal feces, domestic sewage, frozen and non-frozen foods indicate KF and PSE agars to be the most productive. These findings were confirmed by work done at the EPA Narragansett laboratory. For environmental samples (fresh water) good recoveries were noted. PSE and KF gave over 80% confirmation. However, for marine water samples it was noted that one can expect a one log decrease in recovery. It was concluded that additional data would be required to further substantiate this discrepancy and isolate the problem.

At this time, a two-stage testing system was proposed. This proposal was based on a pre-enrichment system with subsequent transfer to M-Enterococcus agar developed by Rose and Litsky. Their data showed significant increases in fecal streptococcus recovery when using this two-stage system. Although increased recoveries were obtained, it was the consensus of those present that two-stage tests may pose additional problems. For example, test results would be delayed because of the pre-enrichment incubation period.

Results therefore, would not be available for three days. Secondly, additional manipulation and equipment would be required. It was agreed that the single-stage test is more appropriate especially where results are available 24 to 48 hrs. after sample processing. Therefore, PSE appears to be the medium of choice, since the incubation period is only 24 hrs. However, the application to a membrane filter technique has not been completed. Work on this phase is in progress at the University of Massachusetts and at the EPA narragansett laboratory. KF agar using the MF technique remains at least for the present time, the medium of choice for processing environmental samples.

The use of the fecal streptococcus test was discussed with the following points being made:

(a) The presence of fecal streptococci in water indicates fecal contamination. However, there are some strains which have limited sanitary significance. These are the atypical Streptococcus fecalis strains which are associated with vegetation and S. fecalis var. liquefaciens which has been found to be ubiquitous. With the latter organism, interpretation of data becomes more difficult when the counts fall below 100 per 100 ml. On the other hand, strains such as S. bovis and S. equinus can be used to denote animal waste or agricultural runoff because of host specificity.

(b) Fecal coliform correlations with fecal streptococci may be useful in determining the source of contamination. Ratios must, however, be applied carefully because of the survival rates of the fecal streptococcus strains. When travel downstream from the point of discharge exceeds 24 hrs., ratios cannot be used. Also, it becomes difficult to establish ratios when fecal streptococcus densities are below 100 per 100 ml.

(c) It was pointed out that the fecal coliform to fecal streptococcus ratios (ratios of 4.0 and above indicating domestic sources while ratios of 0.7 and less indicate animal wastes) were based on fecal samples and not on environmental samples. Ratios developed on actual field samples may not indicate the same results. It was pointed out that ratios may be meaningful and subject to interpretation providing that the following factors be taken into consideration: water temperature, pH, presence of toxic materials, organic nutrients and travel time downstream from the point of discharge.

(d) The use of fecal streptococcus standards to indicate the quality of bathing and recreational waters is not recommended. The following factors discourage its use:

- lack of sensitivity of the medium, especially at a density of 25 per 100 ml. which was proposed by one agency.

- fecal streptococci may survive for a long-time in some waters because of electrolytic content; this persistance may unduly restrict the use of these waters.

(e) Fecal streptococcus data can best be used as supplemental information when run in parallel with the fecal coliform test. The detection of *S. bovis* and *S. equinus* indicates non-human animal pollution. Therefore, identification as to subgroups or species may provide valuable source information. The routine use of fecal streptococcus assays alone is not advocated for recreational waters.

Special Problem

Bacterial aerosols were considered as a special problem. No degree of significance was assigned to the problem due to the lack of information available on the subject. Reports, however, do imply that sewage treatment facilities are potential sources of setting-up aerosols which may harbor pathogenic microorganisms. Work on New York Sewage Treatment Plants indicate microbial emissions into the atmosphere. In conjunction with aeration units, aerosols were found to contain Shigella, Salmonella, beta-hemolytic streptococci, bacillus and acid fast organisms which resemble tubercle bacilli. Concentrations of organisms at various distances away from the plants were not determined. Apparently there is a paucity of data on the dispersion patterns from the treatment facilities. Since there will be a need for such information, methodology will be required. It is recommended therefore that bacterial aerosols be given special attention and methodology for bacterial air sampling be included in the EPA Microbiological Manual.

METHODS FOR DETECTING VIRUSES IN ENVIRONMENTAL WATERS - A STATUS REPORT

Gerald Berg, Ph.D.*

Introduction

Viruses are in essence alive only when they infect, for infection normally results in multiplication and with it the opportunity for the virus to manifest that characteristic of living things - multiplication - and mutation, the only other characteristic of life that the virus is capable of manifesting. Outside of living cells, the virus is inert. Its essential viability in the hostile environment outside the cell is time-marked. Outside of living cells, few survive for long.

Yet, even as their numbers diminish, among those viruses excreted into sewage by infected people, sufficient numbers survive to reach the water intakes of downstream communities. The smallest amounts of viruses detectable in cell cultures, the most sensitive hosts for many viruses, are sufficient to infect susceptible individuals who consume them (Table 1) (1).

Thus, the smallest amount of virus that reaches a water intake or that can be contacted by a recreationalist is a potential hazard. Methods to detect such small amounts must be developed even when detection requires concentrating viruses from large volumes of water.

Over the past several years, a growing awareness within the scientific community of the waterborne virus problem has resulted in the development of a number of techniques for recovering viruses from waters of various qualities. These waters range from sewage to totally renovated. The techniques include membrane adsorption, ultrafiltration, polyelectrolyte adsorption, aluminum hydroxide adsorption, protamine precipitation, two-phase separation, and alginate membrane filtration. Some of these methods appear fairly efficient in limited circumstances. None of them appears to have universal potential at present. The greatest problem, one which may long be with us, is the endless change of the chemical quality of waste and receiving waters, and the unpredictable effects of such change on the efficiency of methods for quantitatively concentrating viruses from water. For this reason alone, the usefulness of standard

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* Virology Section, Advanced Water Treatment Research Laboratory, NERC-Cincinnati

TABLE 1

Infective Doses of Viruses for Man

Virus	Dose	Route of Inoculation	No. Inoculated	% Infected
Poliovirus 1	2 PFU	Oral (gelatin capsule)	3	67
Poliovirus 3	1 TCD_{50}	Gavage	10	30

procedures is doubtful. Methods may always require selection and flexibility to meet the needs of changing situations.

Quantities of Viruses in Environmental Waters

The detectable quantities of viruses in sewage vary from several hundred (Table 2) to ten thousand or more per gallon (2). The numbers generally increase in the warmer months and decrease in the colder months, reflecting infection and excretion patterns in the community. Since viruses do not multiply outside of susceptible living cells, dilution and the toll of time in hostile waters reduce the concentrations of viruses downstream of outfalls to levels barely detectable by the best techniques available even when 50- and 100-gallon quantities of water are tested. Yet these quantities, in terms of the daily water requirements of even small communities, are not small. Table 3 shows the quantities of viruses recovered at or near water intakes of several cities. The data are given for one million gallons of water, an apparently large amount, but in fact only the amount consumed in one day by a community of only 10,000 people.

When one considers that some of the methods used for concentrating these viruses had efficiencies of less than one percent, that the cell culture systems used for detecting the viruses were sensitive to less than 50% of the viruses that are excreted by man, and that there are undoubtedly viruses in sewage that have not yet been detected and identified, it is easy to surmise that the numbers of viruses we now detect are probably several orders of magnitude below the quantities actually present there. Thus, the quantities of viruses that reach intakes downstream of outfalls must be very large indeed.

Recommendations in Standard Methods for the Examination of Water and Wastewater (13th edition) for Methods for Detection of Viruses in Various Waters

Because of its high volume capability and thereby its potential sensitivity, the gauze pad method is recommended for qualitative studies by Standard Methods and the pH 7 membrane adsorption procedure is tentatively recommended for quantitative studies. No recommendations are made for routine examinations of environmental waters.

Methods for Detecting Viruses in Sewage

In the mid-sixties, technology for the detection of viruses in sewage moved from the non-quantitative and

TABLE 2

Concentrations of Viruses in Sewage (1970)

Site No.	1	2	3	4	5
Viruses recovered (PFU/100 gallons)	11,900	38,600	40,500	31,800	13,400

TABLE 3

Recovery of Viruses at or Near Water Intakes

	8/28/70	10/1/70	11/12/70	11/19/70	11/19/70	4/1/71
	(PFU/million gallons*)					
Viruses recovered from water	160,000	40,000	640,000	0	0	0
Viruses recovered from solids	0	20,000	20,000	50,000	20,000	30,000

*Actual samples tested were 50 and 100 gallons and solids from those volumes.

relatively insensitive grab sample and gauze pad techniques to the relatively sensitive and quantitative aluminum hydroxide adsorption, protamine precipitation (3, 4), and phase separation procedures (5, 6).

The aluminum hydroxide and protamine precipitation procedures, as modified in our laboratory, are a combined procedure arranged in tandem whereby aluminum hydroxide gel is mixed with sewage, filtered out, and assayed for adsorbed viruses while the viruses remaining in the filtrate are precipitated with protamine sulfate, eluted, and also assayed. Aluminum hydroxide originally was thought to adsorb only the smaller viruses, the enteroviruses, and the protamine was believed to precipitate only the larger viruses, the reoviruses and adenoviruses. In fact, most of the enteroviruses are adsorbed and removed with aluminum hydroxide and protamine does remove most of the larger viruses, but aluminum hydroxide is now known to remove some of the larger viruses and the protamine is now known to precipitate some of the enteroviruses. The original use of this combination method, one of parallel testing of two sewage samples with each method, clearly must have resulted in high estimates of the amount of viruses present in sewage. The modified system of tandem operation obviates this error.

The aluminum hydroxide-protamine precipitation procedure appears to be an efficient recovery system, but newer methodology has resulted in better virus recoveries.

The two-phase system (5, 6) has also yielded good virus recoveries from sewage, perhaps equal to those reported for the aluminum hydroxide-protamine sulfate procedure. In two-phase, polyethylene glycol and dextran are mixed with sewage and allowed to separate overnight at 4°C into two immiscible phases. The virus concentrates in the lower dextran phase and in the interphase and can be precipitated out and assayed. This procedure may be sensitive and quantitative, but the overnight separation requirement makes it rather cumbersome.

The most sensitive method available today for recovering viruses from sewage is also the simplest (7). Sewage, when the pH is lowered to 3 and filtered through a 0.45 µm cellulose nitrate membrane filter, yields most of those viruses to the filter. The adsorbed viruses are eluted by calf serum in borate buffer (pH 8) when the elutant is passed through the membrane (8, 9).

Other techniques have been less effective.

Recovery of Viruses from Seeded Distilled Water

A membrane filter (MF) method for detecting viruses was first described some years ago (10) and modified to the point where it is 100% efficient for recovering some viruses from distilled water (and perhaps from other very clean water) when volumes up to 25 gallons are sampled (11). The method is one of filtering water buffered at pH 7 (thus adding salt) through a 0.45 µm cellulose nitrate membrane onto which the virus adsorbs, and eluting the virus by sonicating the membrane in beef extract.

Comparative experiments have shown that viruses in distilled water adsorb much better at pH 7 than at pH 3, since much better recoveries are experienced when adsorption is accomplished at the higher pH (Table 4). The presence of organics, which interfere markedly with virus adsorption to the membrane at pH 7, seems not to interfere with adsorption at pH 3 and may actually favor it. Critical experiments to clarify the role of organics in adsorption at low pH are now under way.

Adsorption of viruses onto the insoluble polyelectrolyte PE 60, also interfered with by organics (12), is not an efficient method for recovering viruses from distilled water either (Table 5). Some viruses, such as polioviruses, are recovered with modest efficiency, but other viruses are not. Adenoviruses (not shown in Table) are difficult to recover at all.

Recovery of Viruses from Seeded Tap Water

Efforts to recover viruses from seeded tap waters by the MF and the insoluble polyelectrolyte (PE 60) techniques have yielded erratic results, probably reflecting an erratic chemical composition of the tap waters (Table 6). The pH 7 method resulted in good recovery of poliovirus 1 from one-liter quantities of water in two of three tests, yielding more than 80% of the virus in both. At pH 3, recovery of the virus from one liter of water never exceeded 52% and twice was 5% or less. With 50-gallon volumes, the efficiencies of these procedures were always reduced markedly. Even with the pH 7 method, they never exceeded 44%. In one test, the PE 60 gave poor results comparable to those with the membrane at pH 7 in the poorest test of the series with that method.

There is clearly a long way to go in the development of methods for recovering viruses quantitatively from tap water.

Table 4

Recovery of Viruses from Seeded Distilled Water by MF Technic at pH7 and 3

Virus	Sample Volume	Recovery Method	% Virus Recovered
Poliovirus 1	1 Liter	pH7-MF	104
		pH3-MF	53
	50 Gal	pH7-MF	45
		pH3-MF	17
Reovirus 1	1 Liter	pH7-MF	16
		pH3-MF	115
	50 Gal	pH7-MF	37
Coxsackie-virus A9	1 Liter	pH7-MF	90
	50 Gal	pH3-MF	70
Echovirus 7	1 Liter	pH7-MF	115
		pH3-MF	129
	50 Gal	pH7-MF	51

Table 5

Recovery of Viruses Seeded Into Distilled Water
by Adsorption Onto Polyelectrolyte PE 60

Virus	Control (PFU)	PE 60	
		(PFU)	(% recovery)
Poliovirus 1	75	38	51
Echovirus 7	79	24	30
Reovirus 1	105	33	31
	84	14	17

Table 6

Recovery of Poliovirus 1 from Seeded Tap Water
by MF and PE 60 Technics

Test No.	Sample Volume	Recovery Method	% Virus Recovered
1	50 Gal	pH7-MF	29
		pH3-MF	0
2	1 Liter	pH7-MF	102
		pH3-MF	5
	50 Gal	pH7-MF	31
		pH3-MF	0
3	1 Liter	pH7-MF	84
		pH3-MF	52
	50 Gal	pH7-MF	44
		pH3-MF	36
4	1 Liter	pH7-MF	33
		pH3-MF	2
		PE 60	24
	50 Gal	pH7-MF	18
		pH3-MF	0
		PE 60	18

Recovery of Viruses from River Water

Recovery of viruses from river water by the membrane adsorption method is relatively poor at pH 7 probably because of the organic matter present, but even poorer at pH 3 perhaps because enough organic matter is not available (Table 7). The critical experiments clearly have yet to be done.

The PE 60 method has been used successfully with river waters (Table 8) and has been a major factor in demonstrating the existence of the virus problem, for it was with this method that viruses were demonstrated at water intakes. The few comparative data available with PE 60 and the membrane method indicate that the membrane procedure produces superior results with river water. Here, too, effective methodology is at an early stage of development.

Recovery of Viruses from Ocean Water

In a limited series, the membrane procedure at pH 3 was clearly superior to the PE 60 techniques for recovering viruses from ocean water (Table 9). More viruses were recovered from 15-and 25-gallon quantities of ocean water (all that could be passed through the membranes in these tests) by MF, than from 25-and 100-gallons of the same water by the PE 60 method. The factors in ocean water that affect these recovery systems may hardly be surmised.

Recovery of Viruses from Solids

It has been apparent for some time that much, and perhaps most, of the viruses in the water environment are adsorbed on solids in the water. Generally, the more solids there are in a water the more viruses one can expect to find adsorbed on the solids, although rates of adsorbtion differ for different solids.

In our early efforts, even as we found viruses with equal or greater frequency on the solids in water than in the water itself, and often in greater quantities as well, we found also that our method for recovering adsorbed viruses had an efficiency of less than 1% (Table 10). Clearly, the amounts of viruses adsorbed must have been several orders of magnitude greater than what we were able to recover. At the time, viruses were recovered from solids simply by stirring the solids in 3% beef extract for one hour and assaying the extract after centrifugation to remove the solids.

Table 7

Recovery of Viruses From River Water by MF Technic at pH7 and 3

Sample Size (Gal)	Recovery Method	PFU Recovered
50	pH7	29
50	pH3	1

Table 8

Recovery of Viruses at Selected Sites Along the Mississippi River

	Site No.					
	1	2	3	4	5	6
Viruses Recovered (PFU/50 Gal)	24	76	32	10	30*+	20*

*Recovered from 100-gallon water samples.

+Recovered near water intake.

Table 9

Recovery of Viruses From Ocean Water by PE 60 and pH3-MF Technics

Test No.	Sample Size (Gal)	Recovery Method	PFU Recovered
1	25	PE 60	0
	15	pH3-MF	3
2	100	PE 60	0
	25	pH3-MF	11

Table 10

Efficiency of Recovery of Seeded Poliovirus 1 From River Solids

Grams of River Solids	Virus mixed with solids (PFU)	Virus recovered from supernate (PFU)	Virus recovered from solids (PFU)	% Virus recovered from solids
5	492	78	3	0.6
10	492	28	1	0.3

Efficiency of recovery from solids has improved to about 25% in recent months, but here too we are only at the beginning of the problem.

Current Status of Methodology for Detection of Viruses in the Water Environment

The present status of methodology research in waterborne virus detection is one of concurrent new methods development, improvement of recent and sometimes older methods, and comparative evaluation of the new with the old. Methods available today allow detection of viruses in situations where they could not have been detected just a few years ago.

Yet, we know that we miss a large number of viruses present in the waters that we sample. And we have only the beginnings of an inkling into the essential and comparative effectiveness of methods presently under development. There are many other methods under study than those discussed herein which, although not highly promising at this moment, may see significant development in the future.

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ZOOMICROBIAL EXAMINATION OF WATER: A STATE-OF-THE-ART

Shih L. Chang, M. D.*

INTRODUCTION

The use of zoomicrobes as pollution indicators may seem antiquated in today's approach to develop methodology for a more complete microbiological examination of water and water supplies. The methods that have been used in some European countries, as described in the 2nd edition of the International Drinking Water Standards (W.H.O., 1962), require identification and enumeration of protozoa of different species or genera to which numerical values have been assigned according to the "saprobity system" (1) for their relative significance as pollution indicators.

Such examination, commonly referred to as biological, is very time-consuming and calls for a competent aquatic protozoologist. Furthermore, the "saprobity system" was developed on the basis that different species/genera of protozoa occur in predominance in different stages or zones of recovery of a body of water from pollution by raw sewage. Since only a few cities and small number of small towns in this country have no sewage treatment facilities, and since the 1972 Amendments to the Federal Water Pollution Control Act make it mandatory for all cities and towns to treat their sewage by 1977, any method using criteria based on pollution by raw sewage would soon be obsolete.

Pollution of surface waters by effluent from secondary or more advanced sewage treatment processes produce certain microbiological changes in the receiving water that are indicative of the event because of the qualitative as well as quantitative differences between the effluent-bound microbes and those that are "native" in the aquatic environment. It has been these differences that provide criteria for the bacteriological methods for examination of water.

Zoomicrobes As Pollution Indicators

For the purpose of methodological development the effluent-bound microbes may arbitrarily be placed in two major categories: namely, the fecal microbes and microbes attributed to biological treatment processes.

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* Water Supply Research Laboratory, NERC-Cincinnati.

The fecal microbes include enteric bacteria, viruses and protozoa. The helminth ova are too few and too restricted in geographic distribution to justify their use as pollution indicators. Microbes of any of these three subgroups are indicators of fecal pollution; their presence has direct bearing on the health hazard potential of the water in transmitting enteric infections. Among the indicators of fecal pollution enteric bacteria are the largest in population, of which the coliforms, streptococci and, to a lesser degree, Clostridium perfringens have been used as pollution indicators in water quality control.

The enteric viruses are even more specific as pollution indicators than fecal bacteria. The relative concentration of enteric viruses to fecal coloforms has been estimated at approximately 1:100,000 (2); therefore, very large volumes of water have to be used in virological examination if a reasonable degree of sensitivity is desired. The methods for such examination are sophisticated and complex.

Very little information is available on enteric protozoa in sewage and sewage effluent. In reporting a method for the examination of cysts of Endamoeba histolytica in water in times of disease outbreak, Chang and Kabler (3) approximated the relative concentration of cysts to fecal coloforms in raw sewage under normal conditions is about $1:10^5$. Since sewage treatment removes amoebic cysts more easily than enteric bacteria, the cyst concentration in secondary effluents is likely to be lower. This observation (4) substantiated the later study made in the Chanute, Kansas water supply during an emergency use of reclaimed water. Only one positive culture for E. histolytica and two for intestinal flagellates were obtained from secondary effluent out of a total of ten examinations of five gallon samples of finished water and one gallon samples of effluents from various stages of treatment. It is apparent that the concentration of intestinal protozoa is too low to justify their use as pollution indicators.

The microbial flora in a biological treatment system can be grouped into saprophytic bacteria and zoomicrobes and fungi. They play a role as links in a food chain even in a biological treatment process and are found in large numbers in the secondary effluent. The ubiquity of the saprophytic bacteria in natural waters and soils reduces the usefulness of these bacteria to nonspecific indicators of water quality as ascertained by the plate count method described in the Standard Methods (5).

The microbes that are particularly concerned in this report are the zoomicrobes. They are free-living microscopic animals and comprised of protozoa (ciliates, amoebae and flagellates), nematodes, rotifers, bristle worms and minute arthropods. These organisms are unrelated to fecal pollution; they are found in low numbers in raw sewage but their numbers increase tremendously during the biological treatment process. Even a primary settling with aeration has been found to result in a significant increase in the nematode population (6). As links in the food chain, the larger zoomicrobes, such as ciliates, nematodes, rotifers and bristle worms, increase in numbers at the expense of the small ones, such as flagellates and small amoebae. In a secondary effluent, the larger zoomicrobes are usually found in greater numbers than the smaller organisms.

Zoomicrobes are useful indicators of pollution by secondary effluent. They are normally members of the aerobic fauna in soil and benthos whenever bacteria-food is available and flourish in the biological treatment because of the rich bacterial growth and relatively aerobic environment provided by such a treatment.

On the other hand, the planktonic microbes in non-polluted to slightly polluted rivers, lakes and impoundments are predominantly algae (including phytoflagellates). Most zooplankton are algae-feeders. This composition of the microbial distribution has been confirmed by findings from surveys of algae (7,8,9), rotifers (10), nematodes (11,12), and nematodes and protozoa (13) in surface waters in the United States.

When a secondary effluent is discharged into a surface water the microbes it carries are intermingled with those native in the receiving water The use of secondary effluent flora as pollution indicators would be impractical if the method requires species/genus identification. The concept of their usefulness as pollution indicators was based on a significant quantitative difference between the effluent-bound and most of the native zoomicrobes (13).

The zoomicrobiological method for judging water quality has to be an adjunct to those indicating fecal pollution. In this capacity the zoomicrobial results can supply information for a more complete interpretation of the state of pollution in the aquatic environment. The fecal bacterial or virological results provide a cross-section of the sanitary quality of water. Zoomicrobial results reflect not only the degree of effluent pollution but also the interaction between the effluent and the receiving water. For instance, the very actively-feeding zoomicrobes, such as the ciliates and bristle worms, generally perish and disintegrate in a matter of hours due to the sudden decrease in food bacteria. Presence of these zoomicrobes indicates gross and very recent pollution by secondary effluents. Very low nematode counts associated with moderately high fecal bacteria counts indicate very slow flow-rate which promotes nematode settling. Very low fecal bacterial counts associated with low protozoa counts but high nematode counts indicate that the effluent has been exposed to disinfection. High fecal bacterial counts associated with very low protozoa and nematode counts suggest the occurrence of a number of unusual events such as transient exposure to anaerobiosis. Anerobic conditions are detrimental to the survival of zoomicrobes but not to enteric bacteria or viruses. Other unusual events which may be adverse to nematodes or protozoa are relatively short residence of secondary effluent in stabilization ponds, or presence of animal respiration poison accidently introduced or slowing accumulating in the receiving water. Zoomicrobial findings can provide some useful information on the state of the water quality in its capacity as a carrier of effluent, as well as to pollution indication.

Methods of Zoomicrobial Examination

1. Development of a zoomicrobial pollution index (ZPI).

A formula was proposed for determining the ZPI (13):

$$\mathrm{ZPI} = \frac{A + B + 1{,}000C}{A}$$

where A is the total phytoflagellate count of a sample, B is the total protozoan count, and C is the total nematode count. To make the formula applicable to a wider range of pollutional conditions, C is expanded to cover both nematode and bristle worm counts.

The formula excludes the rotifers and small arthropods and stresses the importance of the presence of worms. Rotifers and small arthropods are present in relatively small numbers in comparison with the planktonic rotifers and arthropods in open waters.

The weighting factor assigned to the nematode count was based on the presence of thousands to tens of thousands of them in secondary effluents (6, 14) and their relatively long survival in the receiving waters and absence in ¼ to one gallon samples of unpolluted or relatively unpolluted waters (11,12,13).

When the water is no more than moderately polluted, the phytoflagellates are present in greater numbers than the protozoa and nematodes, the value for A is larger so the ZPI approaches 1.0. As the degree of pollution increases, there is a corresponding increase in value of B and C and a subsequent increase in the ZPI. Thus, the higher the ZPI the greater the degree of pollution.

The data obtained in a study of the Missouri and Ohio rivers (13, 15) demonstrated a close correlation among the values for ZPI, nematode count, and the total and fecal coliform counts. These results provided the basis for the quantitative relationship between the values of ZPI and levels of pollution and are reproduced in Table 1.

Table 1 reveals a correlation between the total and fecal coliform counts and the values for ZPI at all levels of pollution. The sensitivity of the ZPI is demonstrated by the significant increase in its value at a point several miles downstream from an effluent discharge. Another point of interest is the continuous decrease in both nematode count and ZPI value as the sampling point was moved further and further downstream from the point of discharge, while the total and fecal coliform counts reached a stationary state when the hydraulic conditions became stabilized.

The procedure for determining the ZPI is relatively simple. The 13th edition of the Standard Methods suggests in the Section on Biological Examination the grouping of microbes into categories of flagellated protozoa, nonflagellated protozoa, phytoflagellates and other algal organisms in a plankton analysis. Rotifers are included but nematodes are not mentioned. Values for A and B, therefore, are supplied by the data from a routine plankton analysis. To obtain the value for C, a sample of water of 1/4 - 1 gallon is filtered through one or more 8-10 microm millipore membrane filters and the particulate concentrates, nematodes included, are washed off the membrane filters with a few mls of dilution water. The washings are pooled and transferred to Sedgwick-Rafter counting chambers. Enumeration of nematodes is done under low power magnification.

Table 1. Correlation between Total and Fecal Coliform Counts and Nematode Counts

Distance from point of discharge (miles)	Total coliform count/100 ml	Fecal coliform count/100 ml	Nematode count/gal	ZPI Value
Missouri River				
$<1/2$	15×10^8	29×10^6	140	4.1
3.5	9×10^7	11×10^6	20	2.2
12.3	12×10^4	72×10^2	8	1.5
35.5	5×10^4	3×10^3	4	1.4
45.7	11×10^4	5×10^3	2	1.2
Ohio River				
$<1/2$	45×10^5	16×10^4	96	3.6
47	91×10^3	51×10^2	24	2.6
104	61×10^2	4×10^2	4	1.5
158	89×10^2	5×10^2	2	1.5
174*	26×10^5	48×10^2	20	2.3
222	29×10^2	45×10^1	1	1.5
7 Rivers	110-350**	0-18**	<1	1.1

*Another point of discharge was located several miles above this sampling point.

**These bacteriological data were included in reference (15).

Data from zoomicrobial examination offers information on the extent of pollution, interaction between effluent and receiving water when the interpretation is made in the light of bacteriological data. For instance, a secondary effluent that has had a residence in stabilization ponds would yield a low value for ZPI and very few nematodes due to the rich algae growth and settling of nematodes during the retension period. A stream used as carrier of such effluent would show relatively high coliform counts but very low value for ZPI.

2. Nematode Pollution Index

In the absence of plankton analysis data, the nematological examination can easily be made and the nematode pollution index (NPI) used as a substitute for the ZPI. The nematode count obtained with the procedure described above can be used alone as a quick determination of the pollution level of a surface water. The counts are expressed as the number of nematodes per gallon.

Summary and Conclusion

The use of zoomicrobes as pollution indicators was described and discussed on the basis of historical background and present pollutional conditions. From these discussions a ZPI was worked out from zoomicrobial examination data obtained in field studies made on 2 polluted and 7 clean or relatively non-polluted rivers. For a quick assessment of pollutional condition in a surface water, the NPI may be used as a substitute for ZPI. A procedure for nematological examination of water has been described.

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METHODOLOGY FOR THE ENUMERATION OF PSEUDOMONAS AERUGINOSA

V. J. Cabelli, Ph.D. and M. A. Levin, Ph.D.*

Standard Methods

Two methods are described for the enumeration for *Pseudomonas aeruginosa* in the section on "Tests for Swimming Pool and Bathing Water Places" in *Standard Methods for the Examination of Water and Wastewater* (1). Both appear to be modifications of procedures described by Drake (2). One is a membrane filter (MF) procedure utilizing the "tech" medium containing 9.05% hexadecyltrimethyl ammonium bromide; the results obtained therewith are described as qualitative. The second is a most probable number (MPN) method; it is recommended in *Standard Methods* (1) that all presumptive-positive tubes be confirmed.

Other media for the isolation and cultivation of *P. aeruginosa* have been described by King and Raney (3) and Goto and Bromoto (4). According to Drake (2), most of these media require large inocula and do not yield quantitative recovery of the organism.

Other Methods

Two other methods which are amenable to the examination of environmental and potable water samples have been described. Both are MPN methods. The first is Drake's (2) original method and the second is the *Salmonella* - *P. aeruginosa* method described by Kenner *et al* (5). Drake's method requires confirmation of questionable tubes on an acetamide agar medium; it was examined by Levin and Cabelli (6) and found to be deficient in several regards: recoveries were less than half that on mPA; was not useable with marine waters because a floculate precipitate developed; and the confirmation frequency was poor. The Kenner procedure, probably because of its dual purpose, required that the tubes be streaked on XLD agar (7), followed by confirmation of typical colonies.

The quantity and types of positive data required for the acceptance of any of the four of the above procedures as reference or candidate methods are unavailable from published reports.

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*Northeast Water Supply Research Laboratory
Narragansett, Rhode Island, NERC-Cincinnati

Candidate Method

A membrane filter procedure (mPA) developed by Levin and Cabelli (6) appears to be the best candidate for a reference or standard method for the enumeration of *P. aeruginosa* from recreational and potable waters. The medium (mPA agar) is prepared by adding the ingredients (Table 1) to distilled water, autoclaving the mixture at 121°C for 15 min., cooling the medium to 55-60°C, adjusting its pH to 7.2± 0.1 and adding the dry antibiotics thereto. Minimal quantities of lactose, sucrose and xylose and a pH indicator (phenol red) are included in the medium so that those coliforms which grow in the presence of the inhibitors can be differentiated from the non-fermentative *P. aeruginosa*. The H_2S indicator system permits the differentiation of *P. aeruginosa* from most *Salmonella* and *Proteus* species. The ferric ammonium citrate, sodium thiosulfate and phenol red also are essential for the development of the tan to brown color characteristic of *P. aeruginosa* colonies on mPA medium. The inhibitors used are those to which *P. aeruginosa* is insensitive relative to most Gram-negative organisms. Actidione is incorporated into the medium to prevent fungal overgrowth; the concentration used was shown not to affect the recovery of *P. aeruginosa* from field samples. Membrane filters, through which the water samples are passed, are placed on the surface of mPA agar plates and incubated at 41.5 ± 0.5°C to suppress background organisms including most pseudomonas other than *P. aeruginosa* as well as many of the organisms indigenous to the aquatic environment. Forty-eight hour incubation is required for the appearance of distinctive *P. aeruginosa* colonies. Typically, the colonies are 0.8-2.2 mm in diameter and flat in appearance with light outer rims and brownish to greenish-black centers. The medium is dispensed in 3 ml quantities to sterile 50 x 12 mm petri plates. An agar medium is used since variable recoveries were obtained when liquid media were used to impregnate filter pads. Poured plates of the medium can be stored at 6°C for one month without affecting recovery or selectivity.

Confirmation of typical colonies can be accomplished by the method of Brown and Scott Foster (8), in which the isolate is transferred to a milk agar plate - when transferring from isolated colonies, a single streak on a portion of the plate is sufficient. Following incubation for 24 hours at 35°C, *P. aeruginosa* hydrolyzes the casein and produces a yellowish-green to green diffusible pigment. Verification of colonies typical of *P. aeruginosa* on mPA

Table 1. Composition of mPA medium

Component	Quantity in gm/100 ml
L-lysine HCl	0.5
Yeast extract (Difco)	0.2
Xylose	0.25
Sucrose	0.125
Lactose	0.125
NaCl	0.5
Phenol red	0.008
Sodium thiosulfate	0.68
Ferric ammonium citrate	0.08
Agar	1.5
Distilled water	100 ml

Add ingredients to distilled water; mix, autoclave at 121°C for 15 min.; cool to 55-60°C; adjust pH to 7.1 ± 0.1; and add the dry antibiotics.*

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*Sulfapyridine (Nutritional biochemicals), 17.5 mg; Kanamycin (Bristol-Myers), 0.85 mg; Nalidixic acid (Cal Biochemicals), 3.7 mg; and Actidione, (Upjohn), 15.0 mg per 100 ml or medium.

medium will not be required routinely. However, verification of a number of typical *P. aeruginosa* and other bacterial colonies is necessary not only when an operator is being trained in the mPA procedure but also when the method is used at a new location. The above considerations notwithstanding, in the absence of verification, estimates of *P. aeruginosa* densities should be designated as "probable." Following verification by the method of Brown and Scott Foster (3), the estimates would be considered as "confirmed."

The mPA procedure was evaluated by Levin and Cabelli (6) against the following acceptability criteria: (a) accuracy - the recovery of at least 75% of the "viable" *P. aeruginosa* cells from estuarine and fresh water samples artificially seeded with the organism and stressed by storage in these suspending media; (b) selectivity - the reduction of "backgrouns organisms" in naturally polluted water samples by at least three orders of magnitude (1000 fold); (c) specificity - when assaying field samples, at least 90% of those colonies designated as *P. aeruginosa* should verify as such; and no more than 10% of those colonies not designated as *P. aeruginosa* should, in actuality, be this organism; (d) precision - that, with field samples, the distribution of D^2 value estimates of assay variability, calculated according to Eisenhart and Wilson (9), approximates that expected by chance; and (3) comparability - that the accuracy and sensitivity of the method be equal to or greater than existing methods. The mPA method satisfied all the aforementioned criteria. Subsequent to its development and evaluation, the mPA procedure was used at several other laboratories for the enumeration of *P. aeruginosa* in potable and recreational waters and in sewage samples. It was found amenable to routine use, and confirmation of typical colonies approached 100 percent.

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DISCUSSION

Chang - Did you test the reproducibility of the method by determining the 95% confidence limits?

Cabelli - No, the D^2 method as described by Eisenhart and Wilson was used for this purpose. It is a measure of plate to plate variability across samples.

Chang - Are not 30 samples required for a D^2 analysis?

Cabelli - We were told that 24 would be sufficient.

QUESTION - Did you use the OF test to confirm the identity of suspected P. aeruginosa colonies?

Cabelli - Yes, it was used initially as one of seven tests performed for the confirmation of "typical" colonies. When comparability was established between the results obtained with the seven tests and those obtained by the method of Brown and Scott Foster, the latter procedure was used exclusively.

QUESTION - What do you substitute for the skim milk product used by Brown and Scott Foster?

Cabelli - We used a Carnation skim milk product called "Starlac".

QUESTION - Did you try hexachlorophene as an inhibitor?

Cabelli - No, we did not. Incidently, we did try several chemically defined media with no success.

Geldreich - We are proposing to delete the MF procedure for P. aeruginosa in the present edition of Standard Methods and to substitute the mPA procedure. However, there is still a need for an MPN method for situations in which an MF procedure is not appropriate. What would you recommend?

Cabelli - There are two methods, Kenner's and Drake's. We feel certain both are superior to the one in *Standard Methods*, although we did not examine the Kenner procedure.

Geldreich - There is a need for a round robin to evaluate MPN methods for *P. aeruginosa* for use in situations, such as turbid waters, where MF methods can not be used. We could also examine pour plate methods if such are available.

Cabelli - You are correct.

Kenner - I have evaluated my method on a variety of samples from soils to potable water. In fact, we have used glass fiber filters wherein we could test up to 10 gallons of potable water. Others have tried it with good results.

Geldreich - Jay Vascoucelos found that, in the ground water samples he examined, *P. aeruginosa* generally was isolated when the Standard Plate Count exceeded 500/ml.

Brezenski - Vic, when you examined the samples from the bathing beaches in New York, did you use the mPA method?

Cabelli - Yes

Brezenski - In general, what were the *P. aeruginosa* densities relative to those of the streptococci and fecal coliforms?

Cabelli - We haven't completely analyzed the data; however, they were about 1- 1½ orders of magnitude less.

FUNGI: A State-of-the-Art

Leonard J. Guarraia, Ph.D.*

The outline for the talk, as stated in the agenda, calls for, first, a review of Standard Methods. In the case of the fungi, this is a relatively easy task, since there are no standard methods available. Secondly, a review of candidate methods is called for. Again, this is a relatively easy task, since there are a variety of methods and no clear choice for a standard method, with the exception of the hemp seed bait technique and rose bengal medium which will be discussed later. Finally, suggested recommendations are called for. The most important point here is to develop the techniques and the correlation between specific fungal populations as water pollution indicators.

Fungi are ubiquitous, achlorophyllous, plant-like organisms capable of growing under almost every conceivable condition. In contrast to bacteria, fungi possess a true nucleus and nucleoli, but differ from higher plants in that they do not possess cross walls. Rather the mycelia contain septa which permit the nucleus to float freely within the cytoplasm. Both parasitic and free living forms have been found living in air, soil and water, and will grow on such diverse substrates as preserved specimens in comparative anatomy laboratories, shoes, bread and preserves.

Taxonomically there are four classes of fungi: the Phycomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes or Fungi Imperfecti. Classification is based upon where the spores are formed. Phycomycetes possess no septa and bear endogenous asexual spores. A common Phycomycete is water mold. Ascomycetes develop spores in an ascus or sac-like structure, Allomyces and Neurospora being examples. Basidiomycetes form external spores on a basidium or stalk type structure. The mushroom is a common example. Deuteromycetes are characterized as those fungi for which a completed sexual cycle has not been demonstrated. It is believed that the majority of the Deuteromycetes belong to the Ascomycetes. A more complete review of classification of fungi has been completed by Cooke (13) and Alexopoulos (14). Yeasts are fungi which are said to have lost the mycelial habit of growth and have become unicellular and are found in all classes of fungi.

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*Division of Water Quality and Non-Point Source Control, Office of Water Programs Operations

As relating to water pollution the significance of specific fungi to specific pollutants remains an area open to question. To date there are no data which relate specific general or species to water pollution. Various species are capable of degrading complex compounds such as polysaccharides; cellulose, chitin and glycogen; proteins; casein albumin and keratin; hydrocarbons such as kerosene; and certain pesticides. Most species are aerobic; however, some species are capable of an anaerobic growth.

Fungi occur in all waters and various reviews have detailed their occurrence in both the marine (1-4) and fresh water environments (5). Cooke (6, 7) has described fungi in fresh water streams and rivers. Also, many workers have demonstrated (8-11) that yeasts occur in greater numbers in organically enriched waters than in less enriched systems. The presence of certain yeasts in water has been suggested as a potential indicator of the presence of proteins, hydrocarbons, straight and branched chained alkyl-benzene sulfonates, fats, metaphosphates, and wood sugars (12) by virtue of the fact that the species are capable of degrading these compounds.

Certain microbes have been termed "sewage fungi" based upon their common occurrence in polluted situations and their morphology. One such organism, Sphaerotilus natans is, in fact, a sheathed iron bacterium in the Order Chlamydobacteriales. S. natans characteristically forms chains of cells within a protein-polysaccharide-lipid sheath attached to a substrate. The ability to attach to a substrate provides the organism with a decided growth advantage in flowing streams (16).

Another "sewage fungus" is a phycomycete, Leptomitus lacteus, which produces slimes and flocs in fresh waters. This organism can be found in organically enriched cold waters (5 -22°C) and cannot assimilate simple sugars. Best growth is found in the presence of organic nitrogenous wastes (12). The nomenclature and distribution of the organism has been reviewed by Yerkes (16) and Emerson and Weston (17).

A group of fungi which may more nearly reflect pollution is the coprophilic fungi. These organisms are associated with the feces of animals but rarely of man. Examples are found in the genera Mucor and Pilobolus. These fungi produce spores which attach to plant leaves and are in turn ingested by grazing animals, pass through the alimentary canal, germinate, and use the feces as food. However, few if any fungi are associated with human feces. Sordaria humana has been associated with human wastes under certain conditions (5).

Fungi can adapt to a variety of substrates and require an organic carbon substrate and therefore can be expected to be found in sewage and other organically enriched wastes. As the concentration or organic material increases the number of fungi isolated also increases. This is true not only in water, but also on land; as the organic load of the soil increases the numbers of fungi found also increase (18).

The significance of a specific species of fungi as an indicator of water pollution has not been established. Pathogenic fungi, once introduced into the aquatic environment, are difficult, if not impossible, to reisolate. Associations of enteric pathogens with fecal associated fungi do not have the same meaning as isolation of fecal bacteria (5).

The enumeration of fungi is not equivalent to that of bacteria. The reason for this is that while, with bacteria, one colony usually develops from a single cell a fungal or yeast colony may result from a single cell, from a piece of mycelium, or from a number of cells. Thus no one-to-one relationship exists for easily quantifying the numbers in water.

Sampling for the presence of fungi in the aquatic environment is done in much the same way as for bacteria. Grab samples can be taken using either plastic or glass containers. A minimum volume of 45 milliliters should be collected. Mud, soil or sand samples can also be used. Once the sample is taken, the container should not be tightly sealed (13) so as to permit an exchange of air.

Another technique available to the sampler is the use of baits. A cylinder made of wire mesh stopped at both ends is often used. The bait can be any of a variety of substances, examples being a chunk of beef, apple, rose hip, date and hawthorn fruit. The most common bait used, however, is hemp seed. The bait is allowed to remain in the water for a week and is then returned to the laboratory in a container with the water obtained from the point at which the baits were exposed (13). Once back in the laboratory the baits are placed on the appropriate sterile medium.

Media used for the isolation of fungi are usually similar to those used for bacteria in that there is a carbon source, inorganic salts, agar and peptone. Additionally a dye, usually Rose Bengal, and a broad spectrum antibiotic are included. These are added to prevent or decrease bacterial growth. A thorough discussion of the types of isolation media has been prepared by Cooke (13). The most common fungi found in polluted waters are terrestrial fungi. For this reason the media employed for an aquatic sample are similar to those used for earth samples.

Once growth occurs in the isolation medium, transfers can be made to agar slants. This, however, does not provide any quantitative data. It is possible to isolate many but not all of the yeasts present in the original medium. Distinct colonies can be picked for further identification. Preliminary screening for identification can best be made using a suitable guide such as that devised by Cooke (6), and depends upon knowledge of the spore-bearing structures and general morphology upon staining (13).

The salient points are:

1. Currently there are no fungal indicator species for pollution,

2. Generally an increase in organic material on water is mirrored by an increase in the fungal populations and

3. Present techniques for isolation of fungi give only qualitative answers.

The role of airborne fungal spores in relationship to human health with respect to Coccidioidomycosis, Crytococcocis and Histoplasmosis not addressed in the text is an area requiring consideration.

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SALMONELLA

Donald F. Spino*

INTRODUCTION

This discussion is primarily limited to the isolation and identification of the Salmonella from water. The presentation is in three parts: General review of methods in Standard Methods, 13th edition, available method as a candidate method; and discussion and suggested recommendations on methodology.

Brief Review of Methods in Standard Methods, (13th edition, pp. 697-707)

Standard Methods, when recognizing the presence of Salmonella in the environment, states that the isolation techniques involve relatively complicated procedures that exceed the capabilities of all but a few water laboratories(1). Recent studies have shown that Salmonella can be detected with relative ease in many surface waters(2,3,4,5); one study detected the organism in water that was otherwise of high quality(6).

Primary Enrichment Broth

Tetrathionate broth is among the enrichment broths discussed in Standard Methods. Its excellence for the primary enrichment of Salmonella is noted.

Standard Methods states that when using the tetrathionate broth, incubation should extend beyond 48 hours, with repeat streaking from the same tube daily up to 5 days to ensure recovery of all the Salmonella serotypes that may be present. Stationary growth should be completed between 18 and 24 hours. H. Raj, for example, performed viable counts on 13 different serotypes of Salmonella at 24, 40, 48, and 72 hours(7). For S. typhimurium, the viable count is shown in Table 1(7).

Time	24 hr	40 hr	48 hr	72 hr
Cell Concentration Per Unit Volume	15×10^8	93×10^7	24×10^4	43×10^2

Table 1
Survival of S. typhimurium vs time

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* Solid Waste Research Division, NERC-Cincinnati.

For the remaining 12 serotypes studied there was no increase in the viable count after 24 hours. It would appear that the increased time did not increase the count.

Solid Media (differential)

Brilliant green agar. *Standard Methods* advises that the recovery efficiency of given lots of this medium should be checked with the use of several recently isolated strains of *Salmonella*. Personal observations have shown that if a mixed culture of Escherichia, Proteus, and *Salmonella* is used, then one can observe the efficiency with which non-*Salmonella* are suppressed and *Salmonella* are selected and proliferated. *Standard Methods* states that *Salmonella typhi* and a few other species of *Salmonella* grow poorly on brilliant green agar because of its brilliant green dye content. Difco states that this medium is not recommended for isolation of *S. typhi*(8). For those species that do grow well on brilliant green agar, incubation to 24 hours should be sufficient; in some cases the plates need to stand for an additional 5 to 6 hours at room temperature for full development of some colonies.

Xylose lysine deoxycholate agar. When discussing the color of colonies grown on xylose lysine deoxycholate agar, *Standard Methods* states that after incubation for 24 hours colonies of *Shigella* are red, whereas *Salmonella* and *Arizona* organisms produce black-centered red colonies. Coliform bacteria, *Citrobacter*, *Proteus*, and most paracolons produce yellow colonies. One researcher found that yellow colonies may also be *Salmonella*; if incubated at room temperature for an additional 6 hours (30 hours total) they should turn pink with dark centers(9).

Temperature Options

When discussing the proper temperature for incubation, *Standard Methods* explains how pathogens can be further separated from the surviving nonpathogenic bacterial population with the proper choice of incubation temperature for primary enrichment and secondary diffenentiation on selective solid media. The two factors of temperature and choice of media are interrelated. Great emphasis must be placed on this latter interrelationship.

Biochemical Reactions

For identifying the genus Salmonella, Standard Methods suggests a three-phased schedule. At the end of phase three, 16 tests are required to identify this genus. The candidate method presented here for consideration requires three different media, two reagents, and one antiserum.

CANDIDATE METHOD FOR SALMONELLA IDENTIFICATION

The Hajna Method, with a Modification

In 1951, Hajna found that the reactions for most typical Salmonella are as shown in Table 2(10).

Motility Sulfide Media			Triple Sugar Iron		Broth	"H" Serological
Motility	H_2S	Urease	Slant/Butt	H_2S	Indol	Antigen
+	+	-	alkaline/acid gas	+	-	+

Table 2

Selected biochemical and serological reactions for typical Salmonella.

For this method, three different media, two reagents, and one polyvalent "H" antiserum are needed.

A suspected Salmonella colony may be picked and inoculated into Motility Sulfide Media (MSM), Triple Sugar Iron (TSI), and "H" Broth; incubated 12-18 hours at 35°C. If TSI shows that it is a non-lactose fermenter, then the MSM should be topped with 1 ml of urea broth and incubated at 35°C for six hours. Antigen should be prepared from "H" broth by placing 1 ml of "H" broth culture into an equal volume of 0.6% formalinized saline. The remaining "H" broth culture can be used for the indol test. The culture is discarded if indol-positive. Serological reaction is determined by placing: 0.5 ml of polyvalent "H" antiserum with 0.5 ml of the formaldehyde-treated "H" broth culture, mixed, and placed in a 50°C water bath for one hour. Urease activity is observed on the MSM. Typical reactions of the Salmonella have been given. One may save time by discarding the non-Salmonella earlier than six hours by checking indol production and floculation before reading urease activity.

In 16 water quality surveys of rivers throughout the United States from 1964 to 1972, a total of 317 cultures were identified by this method.* All were sent to the National Center for Disease Control for serological typing. One of them was an Arizona and the remaining 316 were verified as Salmonella of which there were 41 different serotypes.

SUGGESTED RECOMMENDATION

Identification and the Proposed Methods

The simplicity and accuracy of Hajna's method makes it a feasible approach to identifying Salmonella. The simplicity of the technique makes it possible for any technician to do the job in a small laboratory; the 16 tests described in Standard Methods are not needed. Media preparation is reduced by 80 percent when compared with Standard Methods. Urea broth, Kovac's reagent, and the antiserum prepared in 100 ml volumes is sufficient for 200 tests of each. Time for media preparation is saved as well as time consumed for testing.

Media

Enrichment Broths

Tetrathionate, GN broth, and Dulcitol selenite broth are recommended because none are very toxic to the Salmonella and due selectively encourage the growth of Salmonella.

Selective Plating Media

Brilliant green agar. About 90 percent of the colonies that are white with a pink background are Salmonella. The lots of agar should be checked for toxicity with various strains of Salmonella(11).

Galton and Boring(12) state citing references that, The most widely used selective media are bismuth sulfite agar, deoxycholate citrate agar, SS agar, MacConkey Brilliant Green agar, and the brilliant green-phenol red agar.

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*These investigations were performed by the National Field Investigation Center-Cincinnati.

These same authors believe that, "Although Salmonellae may be isolated on all these media, the BG agar, when properly prepared, is more inhibitive for enteric organisms other than Salmonellae, and Salmonella colonies are detected with greater ease than on other selective media."

Brilliant green agar was used as the solid selective medium in the 16 surveys previously noted, with the isolation of 316 Salmonella of 41 different serotypes. The Salmonella found downstrean from a source of fecal pollution, were always recovered on brilliant green agar.

Xylose lysine agar, with the option of preparing xylose lysine deoxycholate (XLD) described by Taylor, is also recommended(13).

Temperature

For isolating Salmonella from the environment, various investigators have used a variety of temperatures:

43.0°C - Harvey and Price(14).

41.5°C - For both the enrichment broths and differnetial plates for sensitivity of isolation of many of the 900 to 1200 serotypes of Salmonellae(3,5,11,15,16,17).

40.0°C - Specifically for the isolation of S. typhi, Livingstone (Johannesburg) personal communication.

39.5°C - Peterson(19).

37.0°C - Could be used in conjunction with elevated temperatures for comparison; perhaps just the first and second days of survey.

Kenner, Dotson, and Smith report an 85 percent recovery of selected Salmonella serotypes at 40.0°C, 25 percent recovery of Salmonella at 41.5°C, and 10 percent recovery at 37.0°C using dulcitol selenite broth (DSE) (7) and xylose lysine deoxycholate plates(18).

One cannot explain successful isolation of Salmonella in terms of temperature alone. There appears to be a marked interdependency of temperature and type of medium used. Livingstone(20) (via correspondence) claims that 41.5°C is too high for isolation of S. typhi. Wun et al., grew S. typhi at 41.5°C in GN broth(20).

Kraft et al(16) states: "The selective inhibitors, sulfathiazole and brilliant green, were added to the tetrathionate broth intended for use at 37.0°C, but brilliant green was omitted from the 41.5°C broth because at this temperature the dye severely inhibited the growth of the live Salmonella species tested."

It is recommended that each laboratory be free to choose a combination of media which would enrich for and selectively differentiate the Salmonella bacteria at incubation temperatures ranging from 37.0°C to 43.0°C. Results should be submitted to an appropriate evaluation committee to arrive at a consensus on methodology.

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AQCL MICROBIOLOGY SECTION REPORT[*]

Herbert Manning, Ph.D.[**]

In the Analytical Quality Control Laboratory at NERC-Cincinnati we have been evaluating enrichment and plating media for detecting Salmonella from natural water samples. Using the gauze swab sampling technique we examined Ohio River sites above and below Cincinnati, as well as a recreational lake.

Our procedure consisted of retrieving the swab after five days, adding it to tetrathionate-brilliant green or dulcitol selenite enrichment media, and incubating it in a water bath at 41.5°C. Rappaport's medium was also included in a split sample comparison with the other two enrichment media. After incubating the primary enrichment broth culture for 7 1/2 hours, a 150 ml volume was added to 150 ml of fresh broth to establish a secondary enrichment culture. At the end of 24 hours both enrichment cultures were streaked to xylose-lysine-desoxycholate, Hektoen Enteric, brilliant green, and MacConkey's agars. These plates were incubated in sealed plastic bags in a water bath at 41.5°C for 24 hours. Twenty typical Salmonella colonies from each medium were picked to TSI agar slants and subcultured to urea, phenylalanine, lysine iron agars, and lactose, dulcitol, and H broths. Isolates with biochemical characteristics typical of Salmonella were then serologically confirmed with Difco Poly A-I O antiserum and Difco Poly a - z H antiserum. Each isolate was also tested with specific O-group antisera. A limited number of isolates from brilliant green agar were identified by CDC-Atlanta and the Ohio Department of Health Laboratory, Columbus, Ohio.

The results, based upon 1,414 isolates, indicated that of the enrichment media tested, dulcitol-selenite was most selective, that is, yielded fewer false positives, and tetrathionate-BG much less selective, that is, yielded more false positives. Preliminary evidence indicates that Rappaport's medium did not enrich for Salmonella when all three media were compared with a split sample. Among the plating media tested the order of decreasing selectivity was found to be brilliant green, Hektoen enteric, xylose-lysine-desoxycholate agar, and MacConkey's agars. The 16 1/2 hour secondary enrichment in tetrathionate-BG broth markedly increased the percent confirmation of Salmonella over that of the primary enrichment. Secondary enrichment in dulcitol selenite broth did not increase the percent confirmation.

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[*] Submitted in writing for the record.

[**] Analytical Quality Control Laboratory, NERC-Cincinnati.

CONCENTRATION OF SAMPLE*

Kathleen G. Shimmin**

Recovery of Salmonella from water is facilitated by employing an initial sample-concentration step. Concentration may occur over several days to a week by suspending a gauze pad into a flowing body of water; after the desired suspension time the pad is placed into enrichment broth and incubated one to five days. Discrete samples (one to two liters) may be concentrated by filtration through either diatomaceous earth or membrane filters (a prefilter may be necessary for waters which are very turbid).

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* Submitted in writing for the record

**Laboratory Support Branch, Region IX

FLUORESCENT ANTIBODY SCREENING[*]

Kathleen G. Shimmin[**]

A fluorescent antibody *Salmonella* screening procedure has been developed for detection of Salmonella in salt and fresh water samples. The procedure basically involves the concentration of bacteria from a water sample in a diatomaceous earth filter aide (Celite). The Celite with the entrapped bacteria is then immersed in *Salmonella* enrichment broth. After incubation of the broth spot plates for primary isolation are prepared. The primary plates are incubated for three hours; then slide impression smears are prepared and stained with a *Salmonella* Panvalent Antiserum (Difco). Positive fluorescence indicates the possible presence of *Salmonella*.

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[*] Submitted in writing for the record

[**] Laboratory Support Branch, Region IX

THE SALMONELLA: DISCUSSION

Geldreich: Dan, I believe that after you reviewed the current edition of *Standard Methods*, you basically came around full circle to saying the same thing. Actually the *Standard Methods*, 13th ed., is a state-of-the-art approach. It has not locked in on any method because there is no one method. Apparently you agree with this. I am a little confused about your objections to *Standard Methods* and what direction you think the methodology should take. You did mention incubation temperature and time. Data as yet unpublished which Fran Brezenski accumulated at Edison, New Jersey indicates there are certain advantages to carrying out the incubation of these organisms beyond the 24 hour limit. Some investigations have proposed as long as five days of incubation. We can't assume that 24 hours is satisfactory.

Spino: I did refer to Raj who performed viable counts on 13 different *Salmonella* species. There was no increase in viable counts after 24 hours. The ability to recover additional species may be related to the volume of sample or enriched growth actually streaked. Limited numbers of selective agar plates are streaked from the enrichment growth and the loopfuls of growth represent very limited sample volumes. Further plate streaking at any time interval may allow the recovery of additional species.

Geldreich: We need flexibility of methodology which could eventually come to the same conclusion reached in *Standard Methods*. We have proposed some limitations in the types of media used. *Standard Methods* has recognized that some media don't recover *Shigella* and has proposed an alternative choice. You don't propose any specific procedure for *Shigella*.

Spino: I did limit my presentation primarily to *Salmonella*.

Geldreich: We have to go beyond *Salmonella* of course; we all agree on that.

Spino: My point was that I felt that these two groups of organisms even though they are in the same tribe, should be treated separately for isolation.

Geldreich: Perhaps the next revision of *Standard Methods* should include a separate section on *Shigella*.

Shimmin: I think the statement Dan made that was different was that each regional or other laboratory should try this range of methods that he suggested and send in the results to a central committee. This is a little different than found in *Standard Methods*. I think it is a good point.

Geldreich: I think this is something we have to do with other media also. I go for that.

Bordner: Methods evaluation is an approach that we don't have in *Standard Methods*. I don't really think *Standard Methods* should be the place for it. An EPA manual that we might develop could promote this kind of evaluation. Also Dan gave us a candidate method, which we asked for, but we don't have to accept it. It is an interesting viewpoint and we could use it as a basis for comparison and method development.

Spino: *Standard Methods* states: "Salmonellae are extremely common in the environment and are probably responsible for most water-borne outbreaks. Unfortunately isolation techniques even for these ubiquitous organisms involve relatively complicated procedures that will exceed the capabilities of all but a few water laboratories." I say that anybody can do this.

Geldreich: I don't agree with you, I think our laboratories are exceptionally high caliber laboratories that can handle pathogen isolation; but this may not be true of small or branch laboratories which use *Standard Methods*. *Standard Methods* covers more than just EPA needs.

Brezenski: To me, "once a pathogen, always a pathogen"; that means that people with certain recognized qualifications must handle them, not just anybody.

Spino: I have shown this identification method to a technician once, and after that he could do it as well as I. I don't think you need a high-powered microbiologist to do it.

Geldreich: This procedure takes more judgment to perform properly than any of the others. One has to have an understanding of all the choices and judgments of the methodology and media, for example the interpretation of colonies on various selective media.

Brezenski: I think what we are driving at is that the methodology per se is a set of mechanical manipulations which are relatively easy and a technician can be trained to do this, but the decision, the choices of media, etc., will have to be made by someone qualified. I use as an example, the scheme which includes motility sulfide medium which is supposed to give you the hydrogen sulfide reaction of the *Salmonella*. I have data and there is some data in the literature showing that the hydrogen sulfide system in that particular medium is not amenable to all the *Salmonella*. For example, we have had to use Sims medium in conjunction because we got negatives with that particular system. If the technician saw a negative H_2S, would he have the knowledge to use the Sims medium or would he disregard it because it is not a H_2S positive strain. There are some H_2S negative *Salmonella* strains.

Spino: I think that if the procedure is spelled out in cookbook form and the technician follows directions, he should be able to do the tests. He could follow all the way through to the "H" antisera floculation tests and check all the reactions.

Brezenski: Look at the manual which NCDC puts out on the Enterobacteriaceae and at the section on *Salmonella* alone. There are many pages devoted to *Salmonella*. NCDC has not restricted their people to specific methods from a clinical point of view, or an ecological point of view. Also in the food analysis section they devote much space to the presentation of different types of media which are available to the technician. Someone has to know that (1) these are available and (2) when to use them. I think we are minimizing this methodology or simplifying it to a degree which we shouldn't be.

Bordner: On the other hand Fran, can you conceive of simplifying the methodology to some extent for our use if we are talking about *Salmonella* from water as opposed to the clinical diagnostic viewpoint where they must get right down to the exact species? Can you see some intermediary position where we could suggest certain choices of media but assure we don't want our microbiologists to go all the way and "count the species" in every case.

Brezenski: We are not doing this now. At the same time I feel we shouldn't be too abbreviated. For example, I have a bone to pick with the philosophy of going to extreme simplicity all the time. I think it is about time we started to add a little more complexity because we have to not because we want to. It requires additional methodology, expertise, and I think our microbiologists are capable of providing this expertise. Let's bring the level up, not down.

Shimmin: Did anyone say anything about serological testing; in the beginning did you mention anything about fluorescent antibody screening?

Spino: No, I did not.

Brezenski: I would like to make a recommendation, if I may. As a candidate method, fluorescent antibody technique for screening should be included. We have enough data available and we are presenting the paper for publication right now. The FDA, I understand, has adopted the procedure and they are going to be using their routine FA technique to screen practically all their food samples and some others. Import samples tested by the Department of Agriculture are to be screened by FA. A technique and conjugates are available from at least three different manufacturers and they are fairly good. Two of these have been evaluated. NCDC has just come out with a publication in *Applied Microbiology* showing that this conjugate works. I definitely feel that EPA should consider the FA as a candidate system and I plan to submit a write-up for consideration.

Shimmin: Have you ever tried the Enterotube? How do you feel about that as another type of identification of *Salmonella*?

Spino: I haven't tried it--I haven't done this type of work recently.

Shimmin: We find it is rather useful for quick and numerous parameters to be tested. We have compared it with individual biochemical media and it worked out pretty well. It is commonly referred to in the literature as well.

Geldreich: Kathy, there are a lot of these methods being released in the literature. I think this is another area where we ought to evaluate a lot of these devices. There are a lot of them that just don't work. People are taking them on faith that they are performing as advertised and again I think our EPA group should really screen a lot of these things out and find what we will accept and what we will absolutely refuse to consider.

Shimmin: I think that is a good idea.

Brezenski: As far as the Enterotube is concerned, I have seen two papers which evaluated the Enterotube system and also the r/b Enteric Differential system. Our laboratory ran them simultaneously with the FA technique and the cultural-biochemical tests; they both showed the same high quality of performance. Let me first say that both these systems are inclined to tell you that you have certain kinds of enteric bacteria present and are supposed to be able to give you enough biochemical criteria to identify the isolate. The systems will tell you if you have a *Salmonella* or not, and this is what we want to know.

Kathy, were you speaking of the improved Enterotube which is relatively recent?

Shimmin: No.

Brezenski: We plan to evaluate the modified one which I think is appreciably better than the original.

Shimmin: At the time we began using it all that was available commercially was the old one. We did test it with test results that were assembled independently and they checked out practically 100%.

Cabelli: I think we are going to have to get numbers (quantitation) of *Salmonella*. Quantitation is needed to fight enforcement cases. Present methods only tell if the sample did or did not contain *Salmonella*.

Jeter: I would like to ask a question of the group as a whole. As several of you know I head up a training unit at Cincinnati and we are always interested in the identification of training needs. This question of *Salmonella* opens up this point loud and clear to you as a group. Over the years we have relied on NCDC to meet the training requirement for identification of *Salmonella*. Is it time for us now to offer training on *Salmonella* collection and identification? If so how far should this be carried? To whom should we address this training? We reach quite a large clientile.

Spino: The candidate method I propose has a simplicity and clarity that anybody who attends the course would be able to use for the identification of the *Salmonella*.

Question from the floor: Can you handle hazardous material in your training program?

Jeter: They are giving botulism training in that same laboratory. I don't think I would hesitate to use *Salmonella* with proper safeguards. Are we talking about incorporating *Salmonella* training in the one course which we call "Current Practices" or should an alternative two or three day workshop specifically be addressed to this one topic?

Brezenski: I think you would have to do the latter because look at the NCDC courses which they give on *Salmonella*. These are one or two week courses. I don't think incorporation into the "Current Practices" is the way.

Jeter: I would agree.

SPECIAL PROBLEMS: DISCUSSION

Kathleen G. Shimmin*

During the course of this seminar a number of special problems were brought up - special subjects which are not currently covered by existing methodology. Topics mentioned include the following:

1. Cultivation procedures for airborne pathogens
2. Special considerations for industrial effluents
3. Cultivation of non-pathogenic organisms (iron, sulfur, photosynthetic, filamentous bacteria, yeasts, fungi)
4. Microbial testing for toxicity
5. Cultivation procedures for waterborne pathogens other than *Salmonella* (e.g. *Shigella*, *Leptospira*, *Vibrio*, *Clostridia*)
6. Rapid serological identification procedures
7. Evaluation procedures for special media and identification systems
8. Evaluation of safety and efficacy of seed cultures and microbiological pesticides
9. Evaluation of automated equipment, electronic counters, video scan systems
10. Investigation of low-temperature bacterial indicators
11. Indicator microorganisms for sludge
12. Evaluation of emergency and field test kits

Excerpts from the discussion period follow.

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*Laboratory Support Branch, Region IX

Special Problems

Borquin: I would like to discuss the impact of seed cultures on the aquatic environment. A recent Atlanta workshop conference addressed this. There are some seed cultures for oil degradation which contain pathogens. Standard testing procedures should be recommended to demonstrate safety and reliability of claims.

Brezenski: With respect to oil degraders, it is time we had a set of procedures to be used on a routine basis to test degradation rates. We had a research topic currently on seed cultures to degrade oil in oil spills. The seed cultures contained Salmonella; hence we couldn't recommend their use. Things like this are cropping up. Tests for biodegradability are being requested by the Permit Program. Should we include such items in our manual or just mention them?

Geldreich: The Advanced Waste Treatment group has looked at biodegradable organisms (especially with respect to detergents). They may be able to supply us with some background material.

Bourquin: I would like to expand the seed culture concern to include the impact of microbial seed cultures on the environment, especially in reference to microbial pesticides (such as those for control of cabbage boll and control of the isopod which preys upon mangroves in south Florida).

Roessler: This probably falls within the pesticide program. In this regard products must be registered for use. There is no standard degradation test set up for new chemicals. The manufacturer must supply data on that new chemical.

Shimmin: How do you evaluate? Do you prescribe a specific set of studies?

Roessler: No. We just require that the manufacturer perform some tests.

Engler: I think two different things are being discussed:

1. Chemicals which are degraded by the environment;

2. Microorganisms which effect this and which may combat this. We should look at spreading and persistence of microbial pesticides (bacteria, fungi, viruses), expecially after they have killed the pest.

Shedroff: Fort Detrick Army Chemical Base was doing testing on chemical warfare. They probably won't give their results, but they might give protocols which might be very appropriate.

Unknown: When with the ultimate disposal unit, I identified the bacteria at three locations: Houston, Illinois, and St. Louis areas. I found that a Pseudomonas sp. broke down oil in oil ponding waste. In work on cutting oils from about ten oil companies, I found that the organism most prominent in breaking down oil was Pseudomonas sp. If organisms were produced in large amounts and dried, perhaps the preparation could be used in breaking down oil slicks; I don't know. We have a paper on that that was given at the International Meeting in 1970.

Lewis: In Richmond, California, at a major Chevron Research Center they have more than 100 different isolates able to degrade various hydrocarbons. We should check with other oil company laboratories. There is a great amount of microbiological study being conducted by these companies themselves.

Brezenski: A lot of money is being spent on this. This is a long-term research. For rapid clean up physical and chemical means to clean up oil spills are now being investigated. They are looking at the biological degraders as something way in the future - a long research program. We're going to have to wait for information to be developed. Let's let this topic lie for the time being until we have more material developed in the next few years.

Shimmin: Do you feel your remarks apply to the entire field of degradation?

Brezenski: We shouldn't give ourselves an undue concern; other topics have higher priority and should be addressed. We can get input on degradation from other agencies. The information is coming slowly.

Shimmin: The purpose of this section is not to put something into a manual but to outline special problems we should be aware of.

Vasconcelos: Standard Methods doesn't address itself to topics such as the single delivery pipet system or rapid plate count techniques, e.g. the video-scan system.

Brezenski: We should give special attention to this. Automatic scanners, electronic counters, and bacterial counters are available now. These have more direct application to us than the degradability topic. Some provision should be made to evaluate these things. Right now it would fall into Bob Bordner's shop; but I'm not sure Bob is equipped to do such evaluation. We are talking about a lot of instrumentation, which is coming up - some of which has almost immediate application to us.

Bordner: I propose that we take up some of the topics which are coming up now and make them the subject of a future meeting similar to the one we have had today. We should establish some types of priorities and then given time for evaluation. Can we consider items for an agenda?

Shimmin: Is there a general interest in this?

Brezenski: Yes, I second that motion.

Shimmin: All right, we'll note that.

Chang: I'd like to mention use of monolayer film, an interest in the early part of the 1960's. I was involved in investigating the cause of degradation. The film was used in water reservoirs and resulted in a great increase of bacterial population. Invariably a

Pseudomonas was involved first. Others would live on byproducts, e.g. *Aerobacter*, *Enterobacter*, *Klebsiella*, or others. These would multiply frequently to very large numbers. I believe now no one in the United States is using this film to suppress evaporation. It is being used in places such as Australia and India.

Gordon: There are two areas which might be considered for future meetings:

1. Indicator microorganisms in sludges:MPN seems to be the answer but recommendations seem to be away from MPN.

2. The plate count in *Standard Methods* is inadequate. It is unrealistic to look at 35°C plate counts when your water only gets to 15°C. What significance is 35°C?

Geldreich: What is in *Standard Methods* with regard to plate counts was meant to apply to potable water and as it relates to interference with indicator organisms. In revision of Drinking Water Standards, we are proposing an upper limit of 500/ml using Standard Plate Count at 2 days, 35°C. I agree with you, Ron. There should be provision for study of some temperatures for environments such as the one you work in. These have a place in an EPA manual, but not necessarily in *Standard Methods*. In *Standard Methods* there should be Plate Count information only on quality of treated water. In the natural environment we have found it is very difficult to interpret a Standard Plate Count and to tie in with some need or some kind of sanitary quality. For these reasons for the past 20-30 years people have been rather cool on the subject. Everything cycles back, so perhaps this should be reinvestigated.

Gordon: I find that organisms which grow at 0°C change in population composition and numbers when you pass through an area of pollution. In the area of Fairbanks, there is a large increase in numbers and change in population types. These organisms grow at 0°C. They don't grow in two days. I grow them for 43 days.

Geldreich: What's the significance of this?

Gordon: The 35°C counts receive wider usage than what they were intended for in Standard Methods. I get at 35°C counts which are very low (e.g. 5/ml). Counts at 20°C and 0°C may be several thousand/ml. I'm not the only one that's got water at low temperature (the upper States of the United States may get down to 0°C). These are important for oxygen consumption under the ice cover. One has to be accurate in streams. You don't have a static population which you can go back and measure. Organisms have to be growing somewhere at these temperatures. You get organisms year round. We don't get sterile streams just because water gets cold. We need to develop methods to look at this methodology which can be used to compare one study with another.

Shimmin: Ed, has the WHO group concerned themselves at all with Arctic conditions for drinking water methodology.

Geldreich: No. There have been a few international meetings on this topic. The one a few years ago in Alaska on waste is the last one I know of. The military has had some.

Gordon: Another meeting which is coming up at the University of Saskatchewan, will be on waste treatment in the Arctic.

Lewis: One part that will enter into this too, when you talk about waste treatment and evaluation of the effectiveness, is the development of some of the new techniques such as the ATP meter (it measures the amount of adenosine triphosphate released upon breakage of a cell and provides rapid identification). I've seen the second generation instrument already, and it's quite probable that this could be developed into an on-line instrument which could give you indication of relative effectiveness of disinfection within minutes of sampling. The technique needs evaluation in comparison to standard quantification methods.

Geldreich: The whole field of rapid methods is a good subject to be included, and many of us are working on it one way or another - ATP & C^{14}, etc. It might be a good subject for our next meeting.

Shedroff: On this I'd like to mention something on permits. Some of the people who are going to have to report on their permits are not going to have all the equipment to do some of these tests. We should consider the use of quick and dirty tests and then a requirement to do something further when the quick and dirty shows up bad.

Geldreich: This gets down to the emergency and field test kits that are on market but have never been approved for *Standard Methods*. The concept might have some limited value but would have to be evaluated.

Lewis: Since I came to EPA, I have worked from time to time on some nuisance bacteria (filamentous forms, iron, sulfur bacteria, *Gallionella*, *Sphaerotilus*, *Crenothrix* and nuisance *Nocardia* forms that can cause trouble in sewage treatment plants). In many cases there are no cultural identification factors or quantitative methods.

Geldreich: We need this not only for waste treatment but also for finished waters and for reservoir waters (e.g. taste and odor organisms). This is very important for early consideration and should perhaps be on the next program. You would be a likely subject to give us some input on the topic.

Lewis: Since *Sphaerotilus* grows so well in sugar beet and pulp mill wastes, instead of trying to get rid of it maybe we could make modifications in systems for separating solids from the liquids and use it in waste treatment.

Chang: Ron, maybe you should mention *Actinomyces* in taste and odor problems, too.

Bordner: The Biological Methods Committee for AIBS is discussing methods for *Actinomyces* and including it in their manual. Does this show a precedent for cross-interests in certain areas?

Knittel: We should consider adopting one key for classification of enteric bacteria [I propose we adopt Ewing's work, because in Bergey's it will be the major one.] We should adopt a standard set of media. During the *Salmonella* discussion many media were mentioned. We should test H_2S production on TSI and should use urea agar for urease production (if broth is used, some weak urease producers are missed such as *Citrobacter* and *Klebsiella*).

Geldreich: I'm going to try in the next *Standard Methods* to have the section on enteric pathogens expanded at least to state-of-the-art presentations.

Chang: *Leptospira* are found in urine. So perhaps you should say "pathogens found in the enteric tract and urine".

IMPACT OF MICROBIAL SEED CULTURES ON THE AQUATIC ENVIRONMENT*

Al W. Bourquin**

The tremendous use of oil for energy in the United States has caused rapid increase in oil imports on large cargo carriers. These large tankers, with capacities equal to or greater than 100,000 dead-weight tons capacity, and increased shipping, has enhanced greatly the danger of major oil spills. With the impending danger of catastrophic spills, technology of clean-up is extremely limited. Present clean-up methods include adsorption and recovery, chemical dispersion, and physical removal. Each technique has limitations due to quantity and type of oil spilled, extent of the slick, and nature of the environment where the spill occurred or where the slick floated. Some authors believe no efficient and safe method exists for clean-up of a spill in shallow estuaries (1,2).

Extensive research is being conducted for the purpose of increasing microbial oil degradation by seeding oil slicks with hydrocarbonoclastic microorganisms. It may be possible that large quantities of selected microorganisms, under proper environmental conditions, could hasten degradation and ultimate removal of pollutant hydrocarbons (1).

The need for standardization of testing procedures for commercially available microbial formulations was pointed out at a recent international workshop held in Atlanta, Georgia. Papers were presented to show that at least two commercial products are completely ineffective or have very little hydrocarbonoclastic activity--below that of natural seawater (3). Other evidence, presented by EPA representatives, demonstrated that at least one commercial formulation contained at least four species of pathogenic microorganisms (4).

A panel, "Environmental Considerations in Microbial Degradation of Oil", at the Atlanta workshop recommended that a committee be formed to study the problems of effective and safe use of microbial seed cultures in the environment. The committee should be composed of members of a governmental agency, members of API--representing the petroleum industry, and members of the academic community who are active in oil pollution research (5).

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* Submitted in writing for the record.

** Gulf Breeze Environmental Research Laboratory, Associate Laboratory of NERC-Corvallis

Microbial seed cultures are currently being studied for application to the environment as microbiological pesticides. Viruses have been isolated which attack selectively the cabbage boll; a bacterium has been isolated as a specific pathogen of mosquitoes; and chitinoclastic bacteria have been proposed as agents against plant predators in estuarine areas. The range of impact on the aquatic environment by seed cultures must be investigated adequately before they are used on a large scale.

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PROCEDURES

COLLECTION AND HANDLING OF BACTERIOLOGICAL SAMPLES: STATE-OF-THE-ART

William J. Stang*

INTRODUCTION

One of the first considerations in an examination of any water supply is the collection and handling of samples. This presentation discusses the current status of Standard Methods regarding the collection and handling of water samples for bacteriological examination. Investigators will agree that the integrity of any sample cannot be maintained if not collected and stored properly. Every research, enforcement, and surveillance effort entails something unique or different about the type of sample desired.

Water Quality Investigations

Potable Water

The 1962 Public Health Service Drinking Water Standards (1) is usually mentioned in any state-of-the-art discussion concerning Standard Methods (2). Responsibility for further development of these standards now lies with an interdepartmental advisory committee that is composed of representatives of various city and state water quality enforcement agencies and representatives of EPA.

The Federal Drinking Water Standards emphasizes collection of samples from representative points throughout a distribution system. Frequency of sampling and the location of sampling points are determined after the quality fo both the treated and untreated water supply has been determined, i.e., the quality of the finished water being controlled, in part, by the quality of the raw water source and, therefore, by the need for treatment.

The monthly minimum number of samples to be examined has been established by the Federal Drinking Water Standards and is based upon the population served by the distribution system. For example, for a population of 1,000-2,000, two samples should be evaluated monthly; for a population of 10,000, twelve samples; for a population of 25,000, thirty samples; and so on, for a population of 100,000, a minimum

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* National Field Investigations Center-Denver,
Denver, Colorado

of 100 samples is required. The number selected from the graph should be "rounded off" in accordance with the following: for a population of 25,000 and under, to the nearest one; 25,001 to 100,000, to the nearest five; more than 100,000, to the nearest ten.

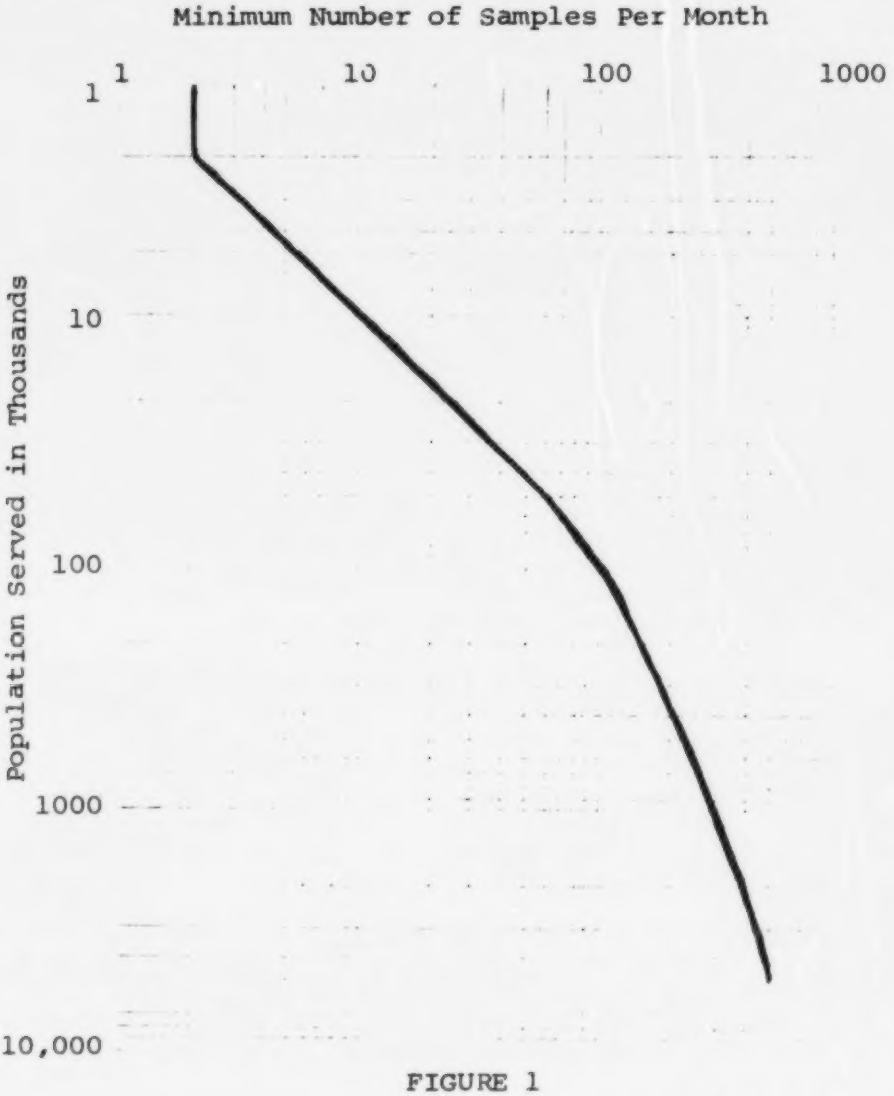

FIGURE 1

Recommended minimum monthly samples per population served by water supply - 1962 Public Health Service Drinking Water Standards

Standard Methods states that it is important to examine samples from widely distributed sampling sites, as well as repetitive samples from any single point (2). Daily samples taken after an unsatisfactory sample has been detected should not be counted in the overall total of monthly samples. Additionally, in the event of an unsatisfactory sample, daily samples should be collected from the sampling point until two consecutive samples yield satisfactory quality water.

The 1962 federal drinking water sampling requirements are now being re-evaluated. It would seem that two samples per month for a city of 2,000 or less population are too few to maintain adequate surveillance of the system. In any surveillance activity of a drinking water system the importance of a well planned and efficient sampling program cannot be over-emphasized. In many cities, the samples are taken from the same locations year after year. These locations are selected for convenience rather than thoroughness, such as a favorite tavern, restaurant, or even the sanitarian's home (3). Samples should be collected from other points that might prove to be a more meaningful measure of the water quality in the distribution system. Additional aspects that should be considered are the qualifications, competence, and integrity of the sample collector. Overfilling sample bottles, inadequate dechlorination of samples, improper sample bottles, prolonged or improper sample transit and storage time are other deviations from Standard Methods that often occur in a water-sampling program (3,4). Therefore, sample collectors should authenticate the samples.

Other variables that might be considered when determining sampling requirements might include: frequency of unsatisfactory samples from supplies serving various population levels, repeat sampling occurrence, and the time interval for repeat sampling. The quality of the raw water supply must be considered; those having poorer water quality should be supervised more closely. When establishing sampling requirements, seasonal population changes, plant capacity, raw water source, and the application of chlorination must be considered (4).

An important subject, concerning sampling of potable water supplies is the continuing development of an extensive evaluation program (4,5). A certifying agency must be responsible for inspecting the laboratory techniques and sampling procedures used by individual laboratories. This combination of internal and external safeguards will provide assurance that sampling techniques, laboratory procedures and equipment will continue to be as sensitive as necessary.

The Technical Advisory Committee on Potable Water Standards is responsible for determining, per unit population, the number and frequency of samples to be collected. Locations of the various sampling points in each municipality are determined by the reporting agency and the regional water supply representative of EPA. The Federal Drinking Water Standards (1) should be closely referenced in any EPA manual that is to be developed.

Surface Water

Surface-water studies are usually efforts to determine source and extent of pollution and are intended either for baseline data collection or potential enforcement action. Representative samples are taken by considering the site, method, and time of sampling. The frequency of sampling is determined by the survey objectives, i.e., does one wish to measure cyclic pollution, duration of peak pollution, or the probable average pollution? State of water quality is measured by collecting samples immediately downstream from the source of pollution. One approach might be an hourly collection during a certain period of time or by advancing sampling intervals one hour each 24-hour period to obtain data for a 7-to-10-day study. To measure state of water quality, samples are collected downstream from mixing zones. Samples from downstream sites are collected less often than those needed for source detection.

Considerations in the choice of bacteriological sampling sites should include base-line locations upstream of the pollution sources, industrial- and municipal-waste outfalls in the stream study area, impact of tributaries, municipal and industrial treatment-plant intake points, and downstream recreational areas affected by certain streamflow time intervals (4). In potential enforcement cases essential consideration should be given to legal requirements.

Cross-sectional sampling or multidepth sampling of the stream may sometimes be necessary in order to determine mixing patterns. Non-representative water areas should be avoided. Another factor to consider in any surface water quality investigation is water use. For example, the water quality at a specific point of water intake or at a restricted water use area is of more value than the average quality of the total pollution loads passing through the cross-section (4). This may be true of source-water intakes, shellfish-harvesting areas, recreational areas or for other restrictive requirements specified for a particular use. Lack of mixing is of no concern in measuring water quality in these situations, and the sampling station is located at the points or areas of actual use. For monitoring stations the frequency of sampling may be seasonal where it relates to recreational waters; daily where it measures raw water used in water treatment; hourly where waste treatment control is erratic; and on a continuous basis for wastewater re-use.

Recreational Water

In the case of swimming beaches or other recreational areas selected sites should include upstream peripheral areas and locations adjacent to natural drains that would discharge stormwater collections, or runoff areas draining septic wastes from boat marinas, or garbage collection areas. Samples of bathing-beach water should be collected at locations and times where the use is heaviest. The optimum frequency during the season would be daily, preferably in the afternoons. Weekends and holidays definitely would be included as periods of highest use. Swimming-pool water should also be monitored during maximum use periods. High chlorine levels in swimming pools rapidly reduce bacterial counts when pools are not in use. Residual chlorine tests are necessary in order to check neutralization of chlorine in the sample.

Data on the water quality of all estuarine bathing beaches should be obtained at high-tide, ebb-tide, and low-tide in order to determine the cyclic water-quality deterioration that must be controlled during the swimming season. Waters overlying shellfish harvesting areas should be collected during periods of most unfavorable hydrographic conditions, most probably at low-tide after heavy precipitation. Procedures for sampling of shellfish and shellfish-growing areas are governed by the National Shellfish Sanitation Program's Manual of Operations (6,7).

Bacteriological sampling stations in reservoirs, lakes, estuaries, and oceans are generally located in grid networks or along transects extending across the long axis of the water bodies. Limited information can be obtained by sampling from the draw-off area of small lakes or impoundments. Samples are usually collected at the same depth over a large area near the outlet.

Municipal and Industrial Wastes

It is becoming increasingly important to sample secondary treatment wastes from municipal waste treatment plants and various industrial waste treatment operations. In situations where the plant treatment efficiency varies considerably, grab samples are usually collected around the clock at intervals for a 3 to 5 day period. If it is known that the process displays little variation, then fewer samples are needed. In no case should composite samples be collected for bacteriological examination.

Sample Transit and Storage Time

Sample transit and storage time are perhaps the most important cautions cited by Standard Methods (2). The bacteria population in a stored sample may change over a period of time. Cold storage minimizes these effects. In circumstances where samples must be mailed, the use of thermos type containers by sample collectors has been suggested (2,8). Maximum use of special delivery or special handling by the postal service must be required. Because most state laboratories and U. S. postal services are minimal on weekends, sample collection schedules should be arranged so as to assure sample delivery to the laboratories at times other than weekends or holidays. This must be a strong recommendation or a requirement in any methods manual that is to be developed.

In the case of secondary treated sewage effluents and industrial waste effluents, where nutrients and/or toxic substances are likely to be present in higher concentrations, the opportunity for bacterial populations to change in a sample is increased. In these types of samples Standard Methods allows a storage time of 6 to 8 hours. The National Field Investigations Centers, working in mobile laboratories can usually process any sample in 4 hours or less.

One consideration that should be presented is the use of commercial airlines or other forms of transit to transport samples contained in ice chests to the laboratory. Often air transport will meet the sample transit time requirements and offset the high costs involved in setting up a field laboratory.

Sampling Devices and Sampling Techniques

Sampling Equipment

Sampling bottles should have a capacity for at least 100 ml of sample, plus an air space for mixing. The bottle and cap must be of bacteriologically inert materials such as glass or heat-resistant plastics. The bottles must be clean, sterile, and contain a proper concentration of sodium thiosulfate so that there are 100 mg/l in the sample. Also, at the time of sterilization the top and cap of the glass-stoppered bottle must be protected from contamination with paper or metal-foil hoods (2).

Most often the type of sample collected is a "grab sample" involving the use of a 250 ml wide mouth bottle. Aseptic technique must be employed to ensure that nothing but the sample water comes in contact with the inside of the bottle or cap.

In sampling from a distribution system, the water faucet is first opened full for several minutes to purge the service line. The flow is then reduced, and the bottle is filled without splashing.

To sample surface water, the bottle is held at the base, pushed rapidly about 6 inches into the water, mouth down, and tilted towards the current to fill. If there is no current, the bottle is moved through the water away from the hand. A small amount is spilled intentionally in order to provide a proper air space (2).

Emmett samplers have been used to collect bacterial samples (9). In this collection device a sterile glass tube is inserted through a one-hole stopper in the cover of the sampler, with the lower end extending inside and near the bottom of the sample bottle. The bottle is filled through this tube, and a fresh sterile tube is used for each sample. The air relief tube extends through the cover into the bucket far enough to reach below the top of the sample so that the sampler stops filling before the neck of the bottle is submerged.

Other sampling devices simply involve placing the bottle in a weighted frame that holds the bottle securely. The bottle is then opened and lowered into the water with a cord or string. Care must be taken not to contaminate the bottle by dislodging dirt or other material from the structure where the collector is standing.

There are various types of sampling devices available where access to the sample point is either difficult, or where depth samples are desired. The general problem is to place the bottle in position at the desired depth, open it, close it, and return it to the surface. No bacteria but those in the sample must enter the bottle.

The most popular depth-sampling device is the J-Z sampler, described by ZoBell in 1941 (10,11). The J-Z sampler includes a metal frame to hold the sample bottle, with a glass tube-breaking device which is activated by a heavy messenger. Either glass or collapsable neoprene rubber bulbs serve as the sample containers. A variation of the J-Z sampler that contains a collapsable rubber bulb is known as the Cobet modification.

Another depth sampler sometimes used is the "Woods Hole" sampler. This device consists of a vane-and-lever mechanism which lifts the cap as water inertia is applied by tugging on the line. As the cap is lifted, water pours into the sample bottle.

The Niskin sampler (sometimes called a sterile-bag sampler or "Book" sampler) is suitable for obtaining 1.5 liters of water aseptically. It can be operated singly, or several devices may be attached in series and activated by additional messengers (12). Other samplers exist with lever or cord attached for pulling the stopper.

The Van Dorn and Kemmerer samplers have been used in deep water without pre-sterilization between sampling. Cross contamination between stations that would significantly alter bacteriological results probably occurs. For enforcement situations involving court litigations this type of sampler is unacceptable.

Peristaltic pumping devices employing sterilizable neoprene or plastic tubing have been used successfully.

Sediment Sampling

Until recently sediment samplers designed strictly for microbiological work were not available. Coring devices with sterilizable plastic liners were the best compromise of sediment samplers available on the market (13). However, contamination with surface water cannot be avoided because these devices must be sent down open. For lack of something more suitable, the Petersen, Ekman, and some shellfish dredges have been used occasionally. However, use of these is not suitable for bacteriological studies in the strictest sense.

A bacteriological sediment sampler designed to collect muds or sediment with use of a sterile, 6 oz. plastic bag is now available--a solution to one very large and serious problem in aquatic microbiology (14).

Sampling for Pathogens

Isolation methods for analyses of water samples for bacterial pathogens usually involve various concentration techniques. Three of the choices available are: the sterile gauze pad placed in flowing water for a 1-to-7 day period (15,16,17) (The exposure time is left to the judgement of the investigator); collection of a large volume sample ranging from one-half to several liters and filtration of the bacteria; centrifugation.

Photographs

Colored photographs are often useful in reports to help not only the general public but the courts as well to better understand scientific material. If photographs are to be used as evidence, these should be well documented on the reverse side. Written documentation should include the signatures of the photographer and witnesses, locations, dates, time, and direction of flow. The photographs should contain suitable annotation and should describe the pollution situation with appropriate landmarks whenever possible.

Survival Studies

Sometimes it is necessary to establish the presence and persistance of coliforms and pathogens. A technique that is becoming increasingly important in enforcement surveys is bacterial survival studies. Microbiologists have employed use of sterile collodian bags in order to entrap a bacterial suspension in situ and remove aliquots of sample from this container for analyses after varied intervals of time. The collodian bag allows small molecules of water and other substances to pass through it, but retains the organisms of interest in an environment (pH, temperature, etc.) very similar to that of the stream.

Another approach is to collect a sample of water, place the sample in a controlled environmental chamber, such as a BOD incubator and examine aliquots for bacteria at predetermined time intervals (18,19,20). The sample may be sterilized and then inoculated with a pure culture of bacteria. This simulated sample is then examined periodically during its exposure to an artificial environment for bacterial survival.

Planning the Stream Study

Workloads imposed on the laboratory facilities and personnel should be planned well in advance of the survey. The principle microbiologist should be consulted in the selection of bacteriological sampling stations as well as the determination of total numbers and types of analyses required.

Chain of Custody

Chain-of-custody procedures should be followed on a sufficient number of samples to prove a water-quality standard violation or a permit violation. For any potential enforcement case, if there is any anticipation of a criminal action, for every sample which may be offered as evidence the chain-of-custody procedures must be followed as a precautionary measure.

The Office of Enforcement now has suggested chain-of-custody procedures (21). Other EPA elements have incorporated these procedures in their own more detailed instructions.

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BACTERIAL SURVIVAL SAMPLER*

Louis A. Resi**

The survival of microorganisms has been important in assessing water quality. Municipal and industrial wastes contain high concentrations of bacterial nutrients, compounds which may be toxic, enteric pathogens, and other microorganisms. Survival studies of waste-associated microorganisms yield additional information in determining the sanitary significance. Rates of die-off and growth have been determined by laboratory incubation procedures. More recently attempts have been made to study bacterial survival under the natural stresses of the aquatic environment.

The Bacterial Survival Sampler is described as having: cylinder body with a screw-cap glass sample port; mounted on each end of the cylinder are 250-ml plastic Millipore MF filter funnels which hold the 47-mm diameter membrane filters, 0.45 um pore size; a metal retainer assembly having fins on one end to give aqua-dynamic stability and maintain the seal of the funnel ends to the body.

Bacterial survival studies are initiated by isolating significant indicator and pathogenic microorganisms from the aquatic environment being studied. Known densities of the sample organisms are inoculated into 300 ml of micro-filtered water. This sample is then placed into the sterile bacterial survival sampler and suspended into the body of water being studied. The sample organisms are retained in the sampler by the 0.45 um membrane filters at each end of the sampler body. The filters allow exchange of the water in the sampler. Aliquots of sample are periodically removed from the sampler through the sample port, and the rates of growth and die-off are determined to show the bacterial survival of the test organisms under the natural stresses of the aquatic environment. The growth-stimulating substances from organic wastes could result in increasing bacterial numbers. Rapidly decreasing bacterial populations could result from toxic wastes.

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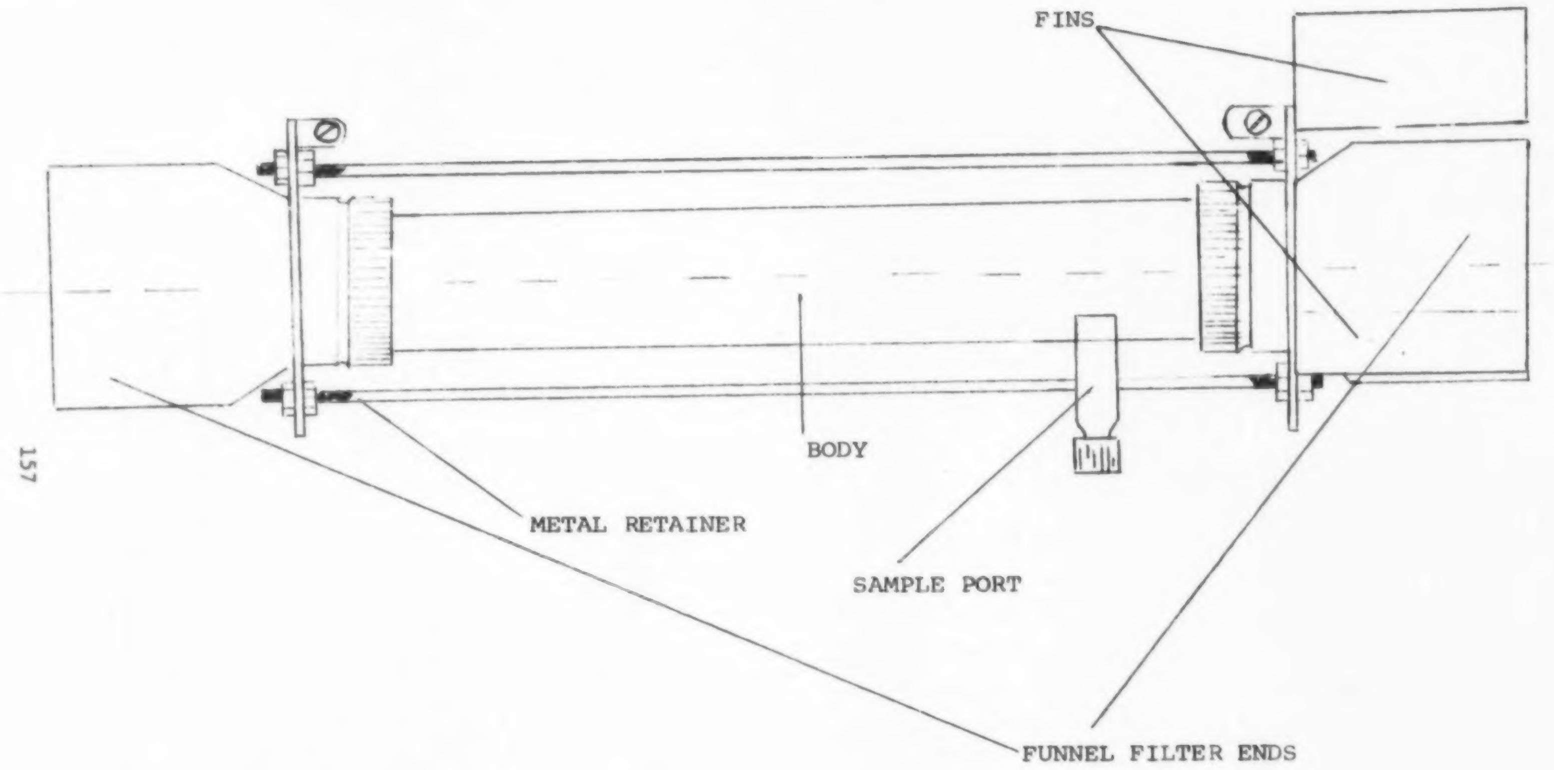
* This paper submitted in writing for the record.
** National Field Investigation Center-Cincinnati.

BACTERIAL SURVIVAL SAMPLER


FINS
BODY
METAL RETAINER
SAMPLE PORT
FUNNEL FILTER ENDS

SEDIMENT SAMPLER*

Edwin E. Geldreich**

The sampler I wish to discuss was developed by Dale Van Donsel and myself several years ago. The mechanics of the sampler will be described in an article to be submitted to Water Research. An advance copy of the publication will appear in the proceedings of the symposium entitled: The Aquatic Environment: Microbial Transformations and Water Quality Management Implications. We have had blueprints made up which are available to those of you who want them.

The sampler involves the use of 6 oz. Whirl-pak plastic bags. The bag is placed over a nose piece and it remains shut until the sample is collected. The sampler is weighted so that it can be lowered to the bottom of a stream or lake. As the sampler contacts the bottom surface, a mud plate releases the mechanism and the bag is opened. As the weight of the sampler bears down, the bag sinks into the mud and is filled. After the sampler has stopped sinking, a double noose that is tied to the bottom of the bag is pulled. This seals the sample in the sterile bag and it can be removed to the surface.

Some advantages of the sampler are that all kinds of bottom materials up to about one inch in size may be sampled. Some pretty large stones will go into the bag if a gravel bottom is encountered. Some of the other samplers now available on the market will jam with anything larger than fine sand.

Different types of mud plates have been tried with this sampler. We have found that if one is working in deep water, a perforated mud plate helps to assure that the sampler will be lowered down straight and will not contact the bottom at a perpendicular angle. In shallow waters a solid plate may be used. Two types of devices are available. With one of them we can attach a rod to the sampler and just ram it down through the shallow water into the bottom sediment. Working from a boat in deep water we attach a double set of cords to the sampler so that it can be released by use of a messenger device. A safety chain is attached so that the sampler will not be lost.

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* This paper submitted in writing for the record.

** Water Supply Research Laboratory, NERC-Cincinnati.

The sampler has worked down to depths of 60 feet in Lake Erie. Its maximum depth is unknown. We do have people studying oceanography who are very interested in the sampler at this time. Dr. Colwell of the University of Maryland is already trying to modify it for her use in ocean studies.

FOERST KEMMERER SAMPLER[*]

For Routine Bay Sampling

Francis T. Brezenski[**]

The kemmerer sampler is cylindrical in shape and is made of brass. It contains two rubber stoppers at either end connected to a rod which passes through the center of the cylinder. It has an automatic device for locking the stoppers open previous to lowering into the water. By dropping the messenger, a mechanism is activated and the valves are closed, thus locking the sample of water. When the unit is closed, the entire weight of the sampler and contents is carried upon the lower valve and therefore is water tight. Under these conditions water from the upper layers are not allowed to enter during the retrieving process. The unit contains an outlet at the bottom which allows for drawing off the water contained in the sampler. This type of sampler has a capacity of 200 ml and is able to collect samples at a wide variety of depths.

Although this sampler has the advantage of collecting a large volume of water at a wide variety of depths and needs no elaborate preparation, it has certain characteristics, which at least theoretically, violate basic bacteriological notions. The Foerst sampler is made of brass and because of the slight solubility of metals in sea water, there may be a bactericidal or bacteriostatic effect on the microorganisms present in the sample. The copper used in the manufacturing process is of prime interest. This becomes even more significant when the water is to be analyzed by a membrane filtration technique. Shipe and Fields (1) reported that samples containing 1 mg per liter or more of zinc or copper compounds interfere with coliform results in membrane filter tests. The metallic ions are adsorbed on the membrane sufficiently to prevent bacterial growth. It is possible, however, that if the sample is collected and transferred quickly to a sterile glass container and the water filtered through the membrane and the membrane immediately placed on the medium, the effect may be minimized. This presupposes excess rinsing of the membrane after sample filtration with sterile buffered distilled water.

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[*] Submitted in writing for the record.

[**] Technical Support Branch, Region II.

Another factor that requires consideration is the use of a nonsterile sampler. The Foerst sampler is not sterilized prior to sampling and is not sterilized after each individual sample is taken. The sampler depends upon a rinsing or flushing type of action for the removal of material that might adhere on to the sampler from the previous sample. When the sampler is lowered into the water it passes through several layers until it reaches the desired sampling depth. During this process, the sampler is in the open position and is being rinsed as it passes through the layers of water. Theoretically, the rinsing action should remove materials clinging to the sides of the sampler from the previous sample. Raritan Bay samples are normally taken five feet from the surface and five feet from the bottom. Therefore, in the shallow sample--the sampler passes through at least five feet of water before it is closed and the new sample trapped in the chamber. With the deep samples, a much more vigorous rinsing is achieved because of the increased depth. The average depth in Raritan Bay is about 35 feet.

The Foerst sampler is used routinely in marine microbiology for qualitative analyses. It has its limitations when assays for total bacteria are required. The most obvious reason for this limitation is the absence of sterile conditions. In the Raritan Bay study, total bacterial densities are not considered. The main consideration, from a bacteriological point of view, is the detection and enumeration of bacterial indices which demonstrate fecal pollution. Due to the nature of the bacterial parameters being used in the study, this type sampler seems feasible, especially when large volumes of water are needed for chemical and bacteriological analyses, (especially when both types of analyses are to be performed on water from the same sample container); where approximately 90 samples are being collected on the same day and where samples are needed from greater depths. The sterile bottle sampler does not lend itself easily to the three demands just mentioned. It was from a point of real need, as imposed by the lack of a practical sampler that this type of unit was approached and studied to see if the problem of mass aquatic sampling could be solved.

The following data are presented on Raritan Bay samples collected simultaneously by the kemmerer and sterile bottle sampler. Coliform densities reported are the average of triplicate plates. Although a limited amount of data was available--it was, however, possible to make several observations.

MF TOTAL COLIFORM/100 ML

Kemmerer(K)	Sterile Bottle(s)	Ratio K/S	Kemmerer(K)	Sterile Bottle(s)	Ratio K/S
11,400	12,000	0.95	2,200	2,200	1.00
11,000	14,000	0.78	560	800	0.70
2,600	1,900	1.36	680	900	0.75
4,500	3,300	1.36	100	400	0.25
8,400	7,800	1.10	2,900	4,400	0.66
5,300	11,000	0.48	23,000	25,000	0.92
540	460	1.17	220,000	350,000	0.62
3,200	4,000	0.80	1,800	1,400	1.28
33,000	110,000	0.30	6,700	4,800	1.40
110,000	180,000	0.61	1,600	1,600	1.00
29,000	28,000	1.03	900	700	1.28
49,000	32,000	1.53	2,400	2,000	1.20
180	250	0.72	45,000	28,000	1.60
170	260	0.65	20,000	24,000	0.83
5,400	5,800	0.93	2,800	4,500	0.62
5,000	4,100	1.21	5,200	7,000	0.74
3,200	3,600	0.88	200	300	0.66
1,200	1,400	0.85	3,800	4,000	0.95
4,500	3,000	1.50	2,400	1,400	1.71
1,500	1,500	1.00	900	1,000	0.90
500	300	1.61	60	90	0.66
120	100	1.20	10,000	9,000	1.11
8,000	6,700	1.19	5,000	4,800	1.04
5,000	4,900	1.02	1,000	790	1.26
26,000	17,000	1.52	80	80	1.00
32,000	26,000	1.23	45,000	27,000	1.66
1,700	2,400	0.70	24,000	23,000	1.04
2,800	3,600	0.77			

		Kemmerer	Sterile Bottle Sampler
No. of samples	=	55	55
Coliform Density			
- Total	=	793,490	1,014,530
Mean	=	14,427	18,446
Median	=	3,200	4,100

Inspection of the data shows that during the study coliform densities of less than 80 and greater than 300,000 were encountered. The median ratio was 1.21 and when the data were ranked according to three density ranges, the following was observed:

Values	Coliform Range	Median Ratio
14	80 - 1000	0.66
26	1300 - 9000	1.11
15	11,000 - 350,000	1.23
55		

In order to check for the possible contribution of copper by the kemmerer through a leaching process, copper assays were performed on bay samples held in the kemmerer for 20 to 30 seconds and 10 minutes.

Analysis for Cu by Atomic Absorption Spectrophotometry

Sample	Holding Time	Cu (mg/l)
1. (Bay Water)	20-30 sec.	0.195
2. (Bay Water)	10 min.	0.195
3. (Bay Water)	20-30 sec.	0.195
4. (Bay Water)	10 min.	0.195
5. (Distilled H_2O)	20-30 sec.	0.075

(Bay water collected in glass container assayed at 0.110 mg/l)

The data indicate that minute amounts of copper are leaching from the sampler. Concentration to 1 mg/l on the membrane does not occur since small volumes of sample water are being filtered.

Conclusions

1. Fifty-two percent of the time, the kemmerer produced values equal to or greater than those received from samples collected with the sterile bottle. The fact that negligible amounts of copper were recovered from water held in the kemmerer enhance the idea that severe toxicity due to metal contamination is not being exerted since concentration values do not approach 1 mg/l.

2. The data in the table are presented in the order that the samples were collected. There is no evidence that carry-over is a severe problem - even when shifting from high to low coliform density areas.

3. At this time there appears to be more variability at the low coliform ranges rather than at the higher ranges encountered in the study. This, however, needs further study

employing a larger number of samples--both at the high and low ranges. Consequently, for this and other reasons, the sterile bottle sampler should be used when lower coliform densities are anticipated; (e.g. approved bathing beach and shellfish harvesting areas.)

4. For the study of Raritan Bay, where gross pollution exists, the above data illustrates that the kemmerer can be used. It is hopeful that a sterile sampler will be available shortly, that will operate at a variety of depths, provide larger volumes of sample water and does not involve elaborate preparation.

5. A continuing comparison of these two samplers be made so as to fully evaluate performance and ascertain limitations at all density ranges.

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BACTERIOLOGICAL VERTICAL WATER SAMPLER[*]

Robert E. Becker[**]

We have used this particular sampler (1) in the Mobile Bay area several times. It seems to work quite well. The sampler can be modified to suit your needs. It has a base plate similar to that of the sediment sampler which was discussed earlier.

The handle of the sampler consists of six foot lengths of galvanized pipe. The end of one pipe section is attached to the base plate. If it is desired, three lengths of pipe may be coupled for longer extension and greater sampling depth. The sample collection device consists of the usual 250 ml sample bottle attached near the lower end of the pipe with stainless steel clamps. The mouth of the sample bottle is sealed with a sterile solid rubber ball. The ball is held in place by elastic rubber tubing threaded through the ball and stretched around the bottom of the sample bottle where it is anchored to the sampler. The bottle is filled by raising the rubber ball from the mouth of the bottle. This is accomplished by pulling on a wire leader attached to the ball and extending to the top of the handle. After filling, the pressure is released, the sampler is raised to the surface and the full sample bottle is recovered.

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[*] This paper submitted in writing for the record.

[**] Water Supply Research Laboratory, Dauphin Island, Alabama.

(1) This sampler was designed and constructed by Clinton A. Collier of the Gulf Coast Water Supply Research Laboratory.

SAMPLING: DISCUSSION

Brezenski: A chamber similar to what Mr. Resi described was being developed for *in situ* bacterial survival studies. On main difference was that the chamber contained an additional port so that access could be made to study changes in temperater, pH, dissolved oxygen and chlorides within the chamber. Of primary concern was the exchange of materials from the outside into the chamber. From the initial--Dr. Litsky (U. of Massachusetts)--found that by using certain dyes and other tracers there was not an adequate exchange of material between the inside and outside of the chamber. The chamber would seem to be selective. Additional criticism of the chamber came from Mitchell of Harvard University who has done many of the marine environment studies, particularly with the indigenous flora of marine waters. Mitchell's whole theory is based on one thing: the die-off and survival rate of the enteric bacteria depends upon the micro-flora present in the water because of the antibiotic type substances that they produce.

Geldreich: Wait a minute, Mr. Brezenski, what we are planning to do is to use a natural sample, and add *Salmonella* to this natural sample. I have to entrap the *Salmonella* in the chamber to study its survival, but the chamber will have other organisms present.

Brezenski: I see, what you are going to do is use "natural" water.

Geldreich: Yes, we didn't make this clear to you.

Scotten: How do you take the sample?

Geldreich: Well, we will anchor it in the water. The chamber has a sampling port large enough to accomodate a 10 ml pipet. The chamber will be taken from the water, sampled from this port, and returned to the lake or stream. The chamber will consist of pyrex glass and other autoclavable materials.

We are in the preliminary stages of testing the device. It is likely that problems will arise that we are not aware of yet.

Cabelli: John Seaburg at the marine laboratory at the University of Rhode Island has developed a device like this to study *Salmonella*. One might contact him. I believe he has solved some of the problems. He has a mixing arrangement inside the chamber.

Geldreich: In a recent publication in *Applied Microbiology* the investigators are using a very small chamber. It has a membrane on each side of it also. Their limited data indicates that the device may work.

Cabelli: Seaburg's device is larger, I believe.

Geldreich: It must be larger for our purposes, because we are sampling over a long period, seven days at least. We are going to have to do additional work on this membrane device.

Kenner: Mr. Geldreich, do you really think that you will get enough exchange of materials between the interior and the outside of the chamber?

Geldreich: Well, we have to investigate the practicality of the device. It may not work, but we will find out.

Shimmin: I have some comments on some things mentioned before we discussed the sampling devices. Based upon data developed in Region II laboratory, basically we do not process any sample from sea water that is older than four hours. We try to analyze the sample within two hours. In the case of samples collected from a fresh water source we do not process samples older than four to five hours. Our studies are planned upon these time considerations.

Gordon: In our area we have trouble with samples freezing. This creates a serious problem which is opposite to that of the usual regrowth or aftergrowth problem encountered if samples are not refrigerated. We know that ice crystal formation kills bacteria, but its not the same from sample to sample, or from one collection time to the next.

What about sterile polypropylene containers?

Geldreich: Yes, those are alright.

Gordon: They are good sample bottles because they are difficult to break.

Geldreich: These are okay as long as they have a screw cap consisting of the same material as the bottle.

Gordon: The cap is polyethylene, and the bottle is polypropylene.

Geldreich: These have been shown to leak after they have been autoclaved.

Gordon: No, these are considered to be disposable. They are discarded after each use.

Scotten: I have seen some evidence in the literature where pure cultures of *E. coli* have been incubated at 10°C and then elevated to somewhere in the neighborhood of 45°C which demonstrated considerable killing of these cells.

Geldreich: Well, when we work on mixed populations we encounter a different set of circumstances than we do with pure cultures. I use pure cultures for a starting point as everybody does. I do not even like to evaluate media with use of pure cultures. I prefer to use natural samples if I wish to study the selectivity and sensitivity of a particular medium. I think that many of the studies in the literature refer to pure cultures. The conclusions may be valid for one particular organism, but they might not work for a mixed population of microorganisms.

Scotten: Well, that is true, of course, I am only pointing out that *E. coli* is susceptible to that kind of treatment.

Geldreich: If anybody would observe this, it would be our people from Alaska. Mr. Gordon, when samples are switched from a very cold environment to the elevated temperature test, do you observe evidence of thermal shock causing a low recovery of fecal coliforms?

Gordon: I have not noticed this to be occurring.

Geldreich: I have not observed data indicating a detrimental affect of thermal shock, except some studies relating to food freezing temperatures with *E. coli* involved; but this is a different environment.

QUALITY CONTROL: A STATE-OF-THE-ART

Robert H. Bordner*

Introduction

An established quality control program is essential to maintain a high level of performance for technical procedures and to ensure reliable and valid data from the microbiological analyses of water and wastewater. Media of substandard productivity, toxic distilled water, incubation temperatures that are too high or too low, nonsterile supplies, and careless recording and reporting of laboratory results are examples of poor quality control in routine laboratory procedures that may drastically affect microbiological data.

Microbiological determinations measuring living organisms that are continuously adapting to a changing environment are not as adaptable to precise, statistical quality control as the analyses performed in other disciplines. Personal judgment is frequently required to determine the next step in a microbiological procedure. Some of the basic tools of quality control in other disciplines such as analytical standards, spiked samples, and quality control charts are not yet suitably adapted to the needs of the microbiologist. Therefore, it is even more important that a high level of control be maintained over laboratory and field procedures and personnel performance.

There is a real need to ensure the quality of microbiological data in view of the increasing emphasis upon water quality standards, enforcement and monitoring activities, and the requirements for evidentiary data in litigation.

Quality Control Programs

All laboratories carry out forms of quality control. Most quality control measures have evolved from common sense practices and the principle of controlled experiments introduced in early bacteriological investigations. Sound practices can be built into a continuous quality control program, from sample collection to data reporting, and conscientiously followed. An effective program should include all the controllable factors that ultimately influence the results. It is especially important that laboratories that only sporadically perform microbiological tests exercise rigid quality control.

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*Analytical Quality Control Laboratory, NERC-Cincinnati

Recently laboratories performing analyses in several different fields of microbiology have reported on the need for and benefits of a quality control program (1-5). Divergent practices and sources of errors were uncovered and improved reliability and reproducibility were accomplished. The Laboratory Division of the National Center for Disease Control (NCDC) and the Food and Drug Administration (FDA) are presently developing formal quality control programs. After thorough review, the proposed procedures will be incorporated into quality control manuals. The NCDC program includes a well-operated proficiency testing system that evaluates performance of over 500 clinical laboratories and a premarket evaluation program for testing serological reagents according to NCDC specifications. The Division of Microbiology, FDA, publishes a Bacteriological Analytical Manual, which is periodically updated. The Division of Microbiology also operates a proficiency testing program for shellfish and food and dairy products utilizing split samples. Microbiology laboratories in the U.S. Department of Agriculture and U.S. Department of the Interior participate in the FDA proficiency testing program.

The U.S. Environmental Protection Agency (EPA) is developing a formal quality control program for microbiology. The development of such a program will be accomplished by a committee of qualified and experienced personnel within the Agency. A good source of quality control procedures that have been recommended for water quality determinations is Public Health Service Publication No. 999-EE-1 entitled Evaluation of Water Laboratories (6), which was originally published in 1966 and is currently being revised.

This presentation, which reflects in part work performed by EPA's National Environmental Research Center (NERC) in Cincinnati, is intended as a review of practices which may be considered for such a program. Some commonly accepted but often neglected measures are highlighted, and other less established practices are proposed.

Sample Collection and Storage

The reliability of results begins with the quality of the sample. A detailed review and full discussion of sample collection and handling was presented in the preceding paper. Sample treatment is an example of many areas where the ideal practice, performing the analysis of the sample immediately after collection, has to be compromised with the practical situation where time is required to transport the samples to the laboratory bench.

Equipment and Supplies

Quality control of laboratory apparatus involves servicing and monitoring laboratory equipment, including the operating temperatures of incubators, waterbaths, hot-air sterilizing ovens, autoclaves, refrigerators and freezers. Thermometers must be checked against a certified National Bureau of Standards thermometer or one of equivalent accuracy. Recording thermometers provide a continuous record of the operating temperatures of test equipment. All temperatures should be recorded on weekly or monthly charts and records should be retained for 2 years. Some laboratories such as the NCDC, Atlanta, Georgia, and Microbiological Associates, Inc., Bethesda, Maryland, have sophisticated remote monitoring systems for controlled environments which are operated from a central control room with an alarm system that sounds when temperatures go beyond the allowable limits (7). Tables 1 and 2 indicate proposed examples of the monitoring of laboratory equipment; the monitoring practices are similar to those used by NCDC (8).

The evaluation of membrane filters is a quality control activity that the Analytical Quality Control Laboratory (AQCL) periodically undertakes. The specifications for the purchase of membrane filters and absorbent pads have for some time included over 20 chemical and physical as well as performance characteristics. Currently there are plans to standardize the chemical and physical tests in the appropriate committee of the American Society for Testing and Materials.

There have been recent reports from EPA laboratories of performance differences in membranes from different sources and of variations in membranes with different lot numbers from the same manufacturer. This type of quality control problem, common to all user laboratories, demonstrates the need for a designated EPA laboratory to continuously monitor such materials and supplies.

Filtering equipment should be kept clean and checked to make certain that it is leakproof and uniformly smooth, and that no toxic metal or corroded surfaces are exposed. Upon one occasion, the chrome-nickel plating covering the bases of about 40 filter funnels the Analytical Quality Control Laboratory had purchased wore off and exposed the brass underneath. All of these funnels were replaced by the manufacturer.

Table 1. Examples of Monitoring Laboratory Equipment

Item	Monitoring Procedure
Balance	a. Check with certified weights weekly. b. Wipe balance and weights clean with ethanol monthly. c. Discard weights if they do not weigh accurately.
pH Meter	a. Compensate for temperature on each run. b. Date buffer solutions when first opened and check monthly with another pH meter. Discard the buffer solution if the pH is more than ±0.4 from the manufacturer's stated pH or if it is contaminated with microorganisms. c. Standardize with at least 2 standard buffers (e.g., pH 4.0 and pH 7.0) before each test or series of tests. d. Have inspected every 6 months.
Spectrophotometer	a. Have inspected every 6 months. b. Check transmittance weekly at a specified wavelength.
Centrifuge	a. Check brushes and bearings every 6 months. b. Check rheostat control against a tachometer at various loadings and frequently enough to ensure proper gravitational fields.
Microscope	a. Clean after each use.
Sterile Air Cabinets	a. Clean filters every 3 months. b. Check for leaks and rate of air flow every 3 months. c. Expose air flow to blood agar plates for 1 hour every month.

Table 1. Examples of Monitoring Laboratory Equipment (Contd)

Sterile Air Cabinets	d. Clean ultraviolet lamps every 2 weeks by wiping with a soft cloth moistened with ethanol. e. Test ultraviolet lamps every 6 months; if they emit less than 70% of their rated initial output, they should be replaced.
Water Stills	a. Drain and clean monthly according to instructions from the manufacturer. b. Clean distilled water reservoir concurrently with the stills. c. Check distilled water continuously against suitable standards.
Dispensing Apparatus	a. Check accuracy of dispensation with a graduated cylinder at the start of each volume change. b. Lubricate moving parts according to manufacturer's instructions or at least once per month. c. Correct immediately any leaks, loose connections, or malfunctions.

Table 2

Monitoring of Controlled Temperature Laboratory Equipment

Autoclave	Each run	a. Also record pressure once during each run. b. Use peak temperature thermometer weekly. c. Use spore strips or spore suspensions monthly. d. If evidence of contamination occurs, check frequently until the cause is determined and eliminated.
Incubators	Recording thermometers and alarm system recommended	a. If recording thermometers are not used, record temperature daily.
Water Baths	Daily before use	a. Clean monthly.
Refrigerators 2-6°C	Daily	a. Clean monthly. b. Defrost or check refrigerator and freezer compartment every 3 months.
Hot Air Oven	Each run	a. Use spore strips or spore suspensions monthly.
Freezers	Daily	a. Connect to alarm system. b. Clean every 6 months.

The test for the suitability of distilled water outlined in Standard Methods (9) is provided to evaluate water from new or repaired stills and as a periodic check on distilled or demineralized water quality. Some laboratories find this test cumbersome and do not run it frequently.

From time to time manufacturers have made special products incorporating the membrane filter or other tests designed for use in field applications. The Millipore Field Monitor and Coli Count Water Tester are examples. In a series of comparisons with the standard membrane filter procedure, the Field Monitor produced a rough estimate of water quality. The Coli Count Water Tester, which has been marketed more recently, did not always yield coliform counts comparable to the count from the Standard Methods procedure when tested at AQCL. However, when representative numbers of blue colonies were picked from the Coli Count for verification, percentages comparable to those from the standard membrane filter (MF) procedure did confirm. The Coli Count will absorb only 1 ml of sample and has been proposed as a rapid field method for preliminary estimates or screening of the sanitary quality of water at new sampling sites. Neither the Field Monitor nor the Coli Count Water Tester are accepted as standard methods. The limitations of these and other specially promoted products should be recognized; data resulting from these products cannot be used for official purposes such as enforcement cases.

Culture Media and Reagents

Culture media should provide good sensitivity, selectivity, and differentiating capability. The use of dehydrated media, when available, is required for more uniform results. Such media should have lot-to-lot equivalence and long-term stability. Purchasers expect to buy a certain amount of quality control when they purchase commercially prepared products. Although some commercial sources state that they routinely test their products with pure cultures of known reactions, these tests may not be directly applicable to water quality analyses and variations from lot to lot are possible. There is a lack of communication between the users and manufacturers; the user's problems and expectations of performance are often not reported to the manufacturer. Media manufacturers indicate an interest in working with the user agencies on improved media standardization.

Prepared media employed in water analyses may be tested with natural samples of the type being investigated and with pure cultures, preferably cultures of representative microorganisms recently isolated from water. The importance of including natural samples in the "use" type of evaluation is emphasized. It is wise, particularly if results from new lots are questionable, to perform a comparative evaluation using as a control a medium lot previously tested and of known capabilities. For example, an evaluation of M-Endo MF medium compares sheen production, colony size, and recovery of coliforms from natural samples, using a previously satisfactory lot as a control.

It is good procedure to test the media prior to and concurrent with use, utilizing cultures with known, stable characteristics. Each laboratory can maintain a collection of positive, negative, and known reaction cultures for the various media used. The amount of inoculum should be standardized by an established technique each time the medium is checked. Stock cultures which have been maintained by frequent transfer, lyophilized, or fast frozen in replicate, are often used. Convenient and standardized reference cultures are commercially available, such as Bact-Check (Roche Diagnostics) or Dri-Bac (Warner-Chilcott Laboratories).

The objectives of media testing are to make certain that nutrient media will support growth from small inocula. Biochemical media must produce the expected positive and negative reactions from control organisms; for example, MR VP tests are checked by Escherichia coli and Enterobacter aerogenes cultures. Differential media are tested with organisms of known reactions to demonstrate the physiological differences among morphologically identical organisms. An example is lactose broth utilized to separate lactose fermenters as pollution indicators from those that are not. When a new batch of these media is prepared, it should be tested with organisms of known fermentative ability. Similarly, enrichment and selective media are tested both for productivity of the desired organisms and the inhibition of others. Brilliant green and xylose lysine deoxycholate agar are examples of plating media that should be tested before use for the growth of typical Salmonella strains and the inhibition of coliforms and gram positive organisms.

Media standardization is an area that needs additional investigation. Published data derived from carefully designed studies are required to evaluate media performance and to define media capabilities related to their use in specific procedures. Also, there are no established specifications for the performance of many selective media.

Among organizations involved in writing specifications or planning media standardization are the APHA Subcommittee on Standardization of Culture Media, the National Committee for Clinical Laboratory Standards, the Interagency Task Force on Media Standardization, the Defense Medical Support Center, the U.S. Pharmacopeia, The National Formulary, The Association of Official Analytical Chemists, and the U.S. Department of Agriculture's Consumer Protection Programs.

The supply and quality of commercial reagents are subject to methodology changes and new knowledge in manufacturing processes. Continuity of reagent quality must depend on compliance of each new reagent with minimum specifications and evaluation of the performance of that reagent compared with previous usage. All reagents can be tested for correct reactions with positive and negative controls before they are used and retested at appropriate intervals. An example is the rosalic acid reagent added to the M-FC medium.

Serological reagents should be evaluated against known antigens and antisera whose reactivity has been confirmed by previous experience. Quality control procedures should be repeated each time reagent batches are prepared, regardless of the expiration date designated by the manufacturer. Unacceptable levels of reagent activity often result from poor storage conditions or from contamination.

The following controls may be incorporated into the test procedures: all reagents should be dated when received and when put into routine use; new lots of reagents should be tested in parallel with old lots of reagents to eliminate any unsatisfactory reagents; positive and negative controls or reference preparations, where available, should be used each time a serologic test is performed; positive controls should be within one dilution of the average titer; if contamination is discovered in sera or antigens, or if the potency has deteriorated, they must be discarded immediately.

Preparation Services

The preparation of culture media must follow prescribed procedures for weighing, measuring, pH adjustment, and sterilization. Frequently, media preparation is in the hands of junior or unskilled personnel; experience, training, and continuing supervision should be provided.

The laboratory should check each prepared batch of medium for clarity, color, pH, and sterility. Growth-promoting capacity may be adversely affected by oversterilization, burning or charring, contamination with metallic salts from unclean glassware, and repeated melting of solid media. Darkening may result from oversterilization. Precipitates may indicate chemical incompatability or overheating. The pH should be taken electrometrically at room temperature and be within ±0.2 units of that specified. If a medium has been overheated at any time, the pH is likely to be lowered. To check sterility, representative tubes or plates are incubated for 2 or more days at 35°C.

Store sterile culture media in a clean, cool, and dark area free from dust, contamination, and excessive evaporation. Many laboratories store media in the refrigerator. Strong light may break down some media such as brilliant green lactose bile broth. Excessive evaporation alters the concentration of ingredients. If changes from exposure to light or evaporation are observed, the medium should be discarded. If media are stored at low temperatures, they must be incubated overnight and any tubes with air bubbles, discarded. Sterile media should be prepared in amounts that will be used within a month.

It is good practice to date dehydrated media when received, rotate in use, store in tightly-closed, screw-capped containers at less than 25°C, and discard if signs of deterioration, such as caking or discoloration, are observed. All dehydrated media in sealed containers should be used within 2 years and thoroughly checked before use. The media should be purchased in amounts that meet the needs of the laboratory for 6 to 12 months. An example is the purchase of membrane filter media in 1/4-pound packages to ensure that the media do not remain unsealed for prolonged time periods before use.

Ideally, an opened container should not be in use longer than 2 or 3 weeks. Such media deteriorate at different rates varying with formulation, humidity, temperature, and other factors. Significant variability in differential and selective plate media resulting from improper preparation and storage have been reported in a laboratory evaluation by Barry, et al (4).

Laboratory personnel should make certain that the glassware cleaning procedures used are adequate and that there is no residual chemical or detergent film. Standard Methods recommends a procedure for the bacteriological testing of glassware for bacteriostatic or inhibitory residues.

Test Procedures

Methods selection is an important application of quality control. A set of guidelines or a procedural manual that describes EPA methodology for microbiological analyses has been suggested. It is reasonable that methods must be clearly defined before a quality control program can be effective. Such a manual would select the best methods for specific applications and identify the options, if options are necessary, that will provide the most reliable and defensible results. Participation and recommendations should come from all EPA microbiologists. The selection of fecal rather than total coliforms, MF as opposed to Most Probable Number (MPN) procedures, and methods for the determination of Klebsiella are examples of microbiological issues for which EPA should establish policy.

Adherence to Agency-recognized procedures is an important factor in quality control. Any deviation from recognized procedures should be carefully noted in the laboratory. A laboratory practicing such digressions should be prepared to demonstrate by quantitative methods that the deviations have not adversely affected test results.

An active program for evaluating new methods and reevaluating the present methods is a function of any laboratory truly interested in improving its performance. Such a program can logically be assigned to quality control.

Quality control also implies the comparison of methods. For example, when changing from the MPN to MF procedure, it is necessary to run a series of samples in duplicate by both methods and to compare results.

Replication of MF analyses is proposed as an aid to quality control. Some laboratories perform all MF analyses in duplicate or triplicate. For research projects using membrane filters some laboratories, including AQCL, filter five replicates for each analysis.

Comparison counts of colonies provide information on precision. Such counts are particularly valuable for the total coliform MF test, in which more than one technician periodically counts the same membranes to reinforce the interpretation of sheen colonies on Endo MF media. At AQCL comparison counts are made on 10% of the membranes.

One of the most frequently used quality control procedures for MF tests is the confirmation of colonies. At least 20 typical, well-isolated colonies for each sample are picked and inoculated into tube media. After incubation the tube media are observed for positive results. The percentage confirmation in tube media is calculated for coliforms from the number of positive tubes. Fecal streptococcal colonies can also be confirmed by inoculation into the appropriate biochemical media.

The confirmation procedure is also used for enteric pathogens isolated from water. Typical colonies from selective media are purified and tested biochemically and serologically. Additional confirmation by NCDC or a qualified state health laboratory is desirable, but usually possible only for limited numbers of isolates. There is a need for EPA to provide this expertise in an Agency laboratory or by contract with an authoritative laboratory such as NCDC.

Comparison of results from field tests with those from the laboratory is necessary. If the delayed coliform MF tests are used in the field, the results should be compared with those from the immediate test.

Data Handling and Validation

Permanently recording analytical data in meaningful, exact terms and reporting it in proper form to some information storage facility for future use implies quality control. Precise rules for the use of significant figures, rounding off of numbers, and arithmetic operations should be agreed upon. An example is the rules to follow for reporting MF results when more than one increment is within acceptable colony count range, but when there does not appear to be a proportionate relationship among the results. A system for maintaining neat, accurate, and legible records and for reporting results is mandatory. Bound laboratory records books and preprinted report forms are recommended. Some programs use multi-copy forms to record all information from sample collection to calculation of results. These forms are then forwarded to the appropriate office for direct transfer of the data to computer programs. Laboratory records should be readily available for inspection and held on file for a period of at least 2 years.

A research group at the Robert S. Kerr Water Research Center at Ada, Oklahoma, has investigated the application of the Cu Sum Quality Control Technique to microbiological results for the purpose of producing a reliable method of validating the data (10).

The conclusions of this validation study were that precision control charts are a useful tool for precision but they cannot measure accuracy; the data can be precise and still inaccurate. The control charts have to be constructed on data from the same waters under study. Tests must be performed at least in duplicate. To obtain maximum benefits, the data must be plotted daily and problems must be rectified immediately.

Performance of Personnel

The specificity and selectivity of microbiological tests can be closely controlled by some of the techniques suggested, but the care with which the test is performed cannot be as easily controlled. Formal training in microbiology and in performing environmental analyses is strongly recommended. The degree of professional training required is related to the number and variety of tests performed. Ideally, the microbiology laboratory should be under the supervision of a professional microbiologist. When this is not possible, personnel performing the microbiological tests should have the readily available support of a professional microbiologist who is experienced in environmental analyses. If the data will be used to support legal action, analyses must be supervised by a professional microbiologist who may be called upon as an expert witness.

On-Site Laboratory Inspection

For years microbiologists in the water supply program have performed evaluations by on-site inspections of laboratories that test the quality and safety of interstate carrier water supplies. Often special problems have been solved, inapparent discrepancies have been brought to light, and more efficient techniques have been developed as a result of this activity. An established laboratory visitation program formally recognizes the capabilities of the laboratory and its personnel. Such a program should be considered for EPA microbiology laboratories.

Intra-laboratory Quality Control

The Analytical Quality Control Laboratory is responsible for providing reference samples to quality control programs in Regional laboratories, NERCs, other federal, state, and local agencies and the private sector. Although this program began with the development of samples for chemical parameters, it includes biological and microbiological parameters as well.

A development of a sample preparation system for microbiological analyses is planned. The problems involved in the development of stable replicate samples are recognized. There is presently no method of effectively preserving water samples for this purpose. The use of replicate membrane filters which have been inoculated, incubated, and dried has been investigated. The rapid distribution of quick-frozen samples and the use of lyophilized cultures are also being considered.

Inter-laboratory Studies

Formal inter-laboratory studies for evaluating all EPA analytical methods for water are also carried out by the AQCL. Stable replicate samples for chemical and physical parameters are provided to participants with exact instructions for sample preparation and analysis within a limited period of time. Results are evaluated and reported in a formal EPA report that provides the precision and accuracy statements of method performance.

For inter-laboratory method studies in microbiology, standardized media and supplies should be prepared in the issuing laboratory and sent to other laboratories for the evaluation of a procedure. A method of sample preservation such as rapid freezing or lyophilization would be required.

An alternative approach would bring the participating microbiologists and technicians together in one laboratory where they could analyze sample aliquots at the same time with the use of the same media, materials, and procedures.

EPA and its predecessor organizations have conducted field tests for the evaluation of delayed total and fecal coliform MF procedures. The microbiology laboratory sends the prepared media and materials to participating laboratories in different geographic locations that perform replicate filtrations for the immediate and delayed tests. The replicates on holding media are mailed to AQCL for completion of the delayed test. Results are compared and evaluated.

Summary

An effective quality control program is the continuous systematic practice of accepted procedures coupled with the correct performance of competent technicians. Such a program, when properly administered, will produce data of uniformly high quality without unduly interfering with the primary analytical functions of the laboratory. A well defined EPA quality control program for microbiological analyses will ensure reliable and valid microbiological data and a high level of performance.

Such a program should include the following:

1. A set of guidelines or a procedural manual that selects and clearly describes EPA methodology with appropriate references to recognized microbiological journals, manuals or books such as Standard Methods and with provisions for periodic updating.

2. An efficient internal quality control protocol in each laboratory to monitor sampling techniques, equipment, supplies, sterilization methods, glassware washing procedures, media, and reagent preparation and data handling.

3. Intra-laboratory methods and performance evaluation programs in each laboratory based on reference samples.

4. A single EPA microbiology laboratory responsible for evaluating and monitoring the quality of equipment and supplies in general use throughout Agency laboratories (e.g., water baths, incubators, field kits, membrane filters and large lots of media for routine tests).

5. An on-site visitation program for EPA laboratories conducted by experienced microbiologists.

6. Participation in all EPA inter-laboratory performance evaluations and method validation studies.

7. Improved EPA specifications for media and supplies.

8. Formal training programs for new laboratory personnel and refresher courses for more experienced technicians.

9. Maintenance of accurate, legible, laboratory data and quality control records.

10. Periodically scheduled meetings of Agency microbiologists to continuously reevaluate methodology and research needs.

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QUALITY CONTROL: DISCUSSION

Geldreich: May I supplement your material by describing the Laboratory Evaluation Program? The program was initially established about 1943 for laboratories that are involved with the examination of public water supplies and to some extent private water supplies. We have through the years developed a systematic approach by going to each of the State Health Department Laboratories and spending a day and a half evaluating their procedures in the areas of sampling, monitoring, laboratory apparatus, glassware, plastic ware, metal utensils, materials and media preparation, culture media, controls on culture media, multiple tube procedures, membrane filter tests and supplemental bacteriological analyses. We take an in-depth look at the laboratory facilities, laboratory management and staff problems. We work right with them at the bench level while they're doing samples and if necessary roll up our sleeves, show them what they're doing wrong and explain how it should be done.

At the end of the laboratory visit we review the evaluation with the laboratory director, the administrator in charge, or his designated representative. We clearly outline any concerns we have, such as deviations in procedures, and suggest improvements; for example, specifications for equipment. The follow-up written report that goes through the EPA Regional Offices to the laboratory director and the state health department officials holds no suprises. This approach has been very rewarding. We've received tremendous cooperation from state health department laboratory people. We have to do this at the state level about once every three years; and at the smaller laboratories it should actually be done every two years. After more than three years the turnover of personnel is such that procedures could change and the quality of the data could deteriorate. Because we cannot begin to cover all the laboratories in this country, we have in turn checked out one individual in each state health department to be the certifying laboratory officer for all water analysis laboratories within his state.

More recently in EPA we have been placing more emphasis on all water supplies, including recreational waters, and looking at the methodologies used in monitoring programs, so that laboratory evaluation has spread out considerably from its original inception.

Some years ago this program culminated in the development of the manual called Evaluation of Water Laboratories. We are rewriting this manual in two volumes, one devoted to chemistry and the other to bacteriology. Associated with the manual is a survey form, a working check list, which we use when we go in to work with laboratories. It covers all the items that we've just briefly discussed here in general and relates to Standard Methods or good laboratory practices. There are some items that we consider good laboratory practices and have been demonstrated to be equal to or better than Standard Methods. In essence, we're trying to put together, chapter by chapter, an opportunity for the user to study his own laboratory and see where he could perhaps use some improvements. We have provided references to original publications in each chapter and have placed a part of this new survey form at the end of each chapter. The reader can expose himself to some of these problems, see what he is doing wrong and hopefully correct it.

We do feel that until Bob and his group can work out a split sampling program, evaluate the media and provide specifications for materials and equipment which we can all take advantage of, the only thing we can do is to visit the laboratories and work with them. We believe very strongly that we need to supplement these laboratory evaluation visits with a split-sampling program. Marty, I think you mentioned yesterday something in this area. Would you like to comment?

Knittel: These are some comments on experience in the aerospace industry. In their quality assurance program, in building a space craft and specifically in the planetary quarantine program in which I was involved, each item had to be checked by another individual whom we called a quality assurance officer. All quality assurance information had to be logged in each day. It was not enough to state that media had been sterilized at 121°C for a certain length of time; they wanted proof that it was indeed sterile. Related to some of the quality control principles that Bob pointed out, procedures were practiced such as obtaining selected portions of a batch of prepared media and actually incubating it to prove that it was sterile. Autoclaves were checked periodically. We used a lot of laminar flow hoods which had to be certified at different points in time. We were dealing with very low levels of contamination on space craft surfaces; we would get perhaps 10 or 20 microorganisms per square inch of surface. When a person was actually involved in doing plate counts or similar determinations, he was indeed following the procedure set forth and someone would be logging in all of this information.

Geldreich: I have one question, Bob. You and I have had this discussion privately, but for the record I would like to say that I am always nervous when we have to concentrate our evaluation of media primarily on pure cultures. I don't believe that you can adequately evaluate a medium only on pure cultures. A greater emphasis should be placed on using natural samples. My reasoning here is that it may be that you have an organism that can do tricks for you and will produce gas or whatever the characteristic is; it works great when you're checking it out on that medium. However, in the real world we're working with mixed floras and you have to not only know something about survival rates and whether the microorganism gives gas or not, but also how it interracts with other organisms. So, I would like the group here to understand that I think we shouldn't put all our marbles in evaluations with pure cultures. We need pure cultures to a certain extent, but we must relate more to natural samples and evaluation of media.

Berg: Can we apply laboratory quality control methods other than laboratory evaluation?

Geldreich: Can we? We wish we could. Some years ago, Gerry, we tried to get into a media certification program. Harold F. Clark, Luther Black and several other people actually had a program started but the lawyers in the DHEW said at that time that federal agencies could not get involved in this type of a certification program; yet NCDC has come up with some arrangement.

Berg: NCDC is the only one?

Geldreich: And they're federal. We need to know the procedure, find that mechanism and use it.

Bordner: It was explained to me at NCDC that the certification program for serologicals is voluntary. NCDC asks the various manufacturers to voluntarily submit samples of every lot number that they put out for NCDC evaluation. The manufacturers don't have to do this and some of them don't; but if they do, NCDC evaluates the products and publishes the results. NCDC also publishes the test procedures.

NCDC finds then that people in the state health and other laboratories who are using these products will check the ratings of the products evaluated. The manufacturers find that it is greatly to their advantage to have their products tested because the state health representatives and other user laboratories check NCDC's evaluation list and a great many of them call NCDC for such information.

Geldreich: We need something in our own organization. If you're doing all of this work, I would very much like to see some of this data so that I can disseminate it and use it myself, and I don't see this. The data you showed today is the first time I've ever seen anything like this.

Bordner: What was that, Ed?

Geldreich: Well, data that you have on evaluations, particularly those on your replicates. I think all of us here need to have this kind of information; you should supply it through some mechanism such as your Newsletter.

Shimmin: There's one point that bothered me both in your presentation today and yesterday in Bill Stang's presentation; perhaps I'm not understanding it correctly. You said today that in field studies there are some laboratories that only run one filter per sample because they have a problem running any more, and yesterday Bill Stang implied at one point that the microbiologist just has to take more or less what happens along. I think that we should really make an effort in this manual to stress the fact that the microbiologist should be involved in planning studies. The microbiologist should be completely in control of what kind of samples are coming into his laboratory, so that if he has to adjust the numbers of samples in order to get adequate quality control, he can.

Geldreich: Is Andy Sidio here? This will be a chance for him to make a comment. You know this is a problem we have with engineering groups, getting together.

Shimmin: I know that. I've not been with EPA very long and I certainly had the problem when I first came, but microbiology in Region IX doesn't participate in a study unless they have been involved in planning it and regulating the frequency of the samples and the timing of the samples.

Geldreich: Lou, would you like to say something?

Resi: Well, my comment is that this is the ideal situation; what you say is being idealistic rather than a realist.

Shimmin: Well, it might be idealistic for the rest of the country but it's not for Region IX.

Stang: I would like to say that at NFIC, Denver, we do get in on the planning and lots of times there are other things involved like the amount of time and money that can be spent. Of course, an engineer is going to ask for the most samples that he possibly can in the shortest amount of time; this is a continuing fight that we have.

Shimmin: Yes, I think that's true, but we should be definite in this manual and say just precisely what kind of errors we're talking about. If we're taking our time giving an inaccurate report of data, then why waste our time doing it?

Bordner: It seems to me that we could come up with some better precision data than I'm familiar with now for duplicates or triplicates or whatever number is required, maybe as a result of the fifty replicate date which AQCL has produced or some other source data, we could say to the engineer, "this is the amount of precision we can give you for triplicates as opposed to single determinations. It will cost extra time or perhaps less overall samples will be examined." We need statistical data to prove the advantage of replicates. How do you feel about that?

Shimmin: Yes, I think that's true but, certainly the chemists are asked how many samples they can run in a day and they add their quality control needs. They feel that maybe they can run this many samples and also they can say this is the amount of time the samples can be stored; if you would exceed that amount of time, we can't do it. So if that's good enough for the chemists, it ought to be good enough for us.

Bordner: Except that they have better quality control data for most of their determinations than we have. So you show the engineers these duplicate or triplicate analyses and you're going to come up with a pair that is split pretty far apart once in awhile and the others are close; is the engineer going to say, "I don't think this is worth it."

Shimmin: Well, I don't think the engineer should be making that decision. That's a microbiologist's decision and the laboratory's decision.

Bordner: I think it's a combination.

Geldreich: Andy, when you plan a survey, and you have a certain amount of bacteriological work to be done, how do you go about making a decision on what you're going to do? The microbiologists have asked a question here. They don't always have an opportunity to say, "we can do this much," but, the engineering section says "we need more than that" and there's just a limitation to what the microbiologist can do. How do you resolve your problem?

Sidio: First of all I think that anybody that sets up a survey and doesn't consult each of the chemists, biologists, and microbiologists, prior to the starting of the study, is making a big mistake right off the bat. First of all he's doing a poor job of planning that study. That is part of working with those people, using them as your consultants. I don't care whether its an engineer planning the study or whoever it might be. There is no one who knows every consideration that is to be involved in that study. Whoever is planning it must deal with microbiologists and other professional people to assist him in planning that study.

It is true in many cases that the person planning that study may say, "I need this much bacteriological data," or whatever it might be. Let's take an example: you tell us you can only take 20 samples a day and we've got to have 40. We have to do one of two things; we either double the crew that we've got, double the facilities, or we double the length of time that we need to get the data. We might put in a ten or twelve hour shift for a period of two weeks or whatever the period of time is. This is part of proper planning of a survey. There has to be a mutual understanding that you're in this thing as a team.

You have to do the survey as a team; you have to work together. You as a bacteriologist cannot go out and do a field study by yourself. You contribute to a team effort. The better the people work together, the better the study and the better the results. The smoother the working relationship together, the better the ability of that group to get a job done. For the past few years we have even taken the attorneys into account. There has to be consideration now that every one of those samples may wind up in court. We now have to bring in that legal member of the team. The whole point is teamwork.

Shimmin: Andy, I'd like to ask you a question--how well does this cooperative effort work at NFIC, Cincinnati?

Sidio: If we have had any success at NFIC, and I'm sure this is true of any other group, I think it is because we have a team effort there.

Shimmin: Do you always involve everyone who's going to be in on a study in the planning of it?

Sidio: There are certain instances where you get a call and you have to move immediately, such as the floods last fall in Pennsylvania. They flew a C124 in from Utah to fly one of our bacteriology labs into the Wilkes-Barre-Scranton Airport. In a situation like that you don't do a lot of planning before you start acting. Usually, however, each of the crew members is included as part of the planning. You can't plan a field study from behind a desk. It's very important to know what you're planning a survey for and to have all of those people there. All of them? No, but all of those people who are going to have responsibilities out there and that includes the field crew that is going to collect the samples. Every person who is part of that field crew is just as important as any other person, nonprofessional as well as professional. Everyone has to know he is an important part of the team. I think this teamwork approach in Cincinnati is excellent.

Jeter: I can vouch for this, Andy. I have been pressed into service with the predecessor organizations of NFIC where we actually sat down around a table and I noticed that every sample collector, and everyone else who was going out on that survey, was around that table and spoke very freely about their responsibilities. I saw it actually working under those field conditions. I can support this very strongly.

Lewis: Bob, I just wanted to bring up the fact that for hospitals and clinical laboratories, unknown specimens are sent out by NCDC and other laboratories. FDA in their milk program does use split samples.

Bordner: Yes, they do. I'm not too familiar with the previous ones in milk. As I understand the program, they've put out split samples using pure cultures and mixtures of pure cultures in milk samples which they sent out frozen. More recently they wanted to get closer to food and they used mashed potatoes. I believe they send out mixtures of cultures for which they can anticipate a certain die-off rate for the cultures they're using. They ask the participating laboratories to warm up and test the samples all at the same time and then return the results. Whether this is practical for a water quality program I don't know.

We've talked a little about round robins in some of our previous discussions. This may be a possibility if we're talking about sending out a medium which is controlled from some central source to be used in participating laboratories with their natural waters and then the results sent back to Cincinnati for evaluation. Does anybody else have any ideas on a round robin or reference sample study? (No comments)

SUMMARIZATION

MICROBIOLOGICAL RESEARCH NEEDS

Kenneth M. Mackenthun*

I accepted this assignment with certain trepidations that, as a result, I too might inhabit the proverbial pitfalls of professional pronouncements. As these are related to research needs, they include:

1. Casting every unknown solution to conceived problems into research needs regardless of their national priority relevancy, or reasonable probability of successful attainment;

2. Displaying noticeable naivete of literature or state-of-the-art as they may be associated with a particular need concept; and

3. Fostering trivia over priority needs by assuming that by mass of need numbers, or by repeating a particular need in a number of different sentence structures, some of the suggestions will stimulate action. Perhaps I may stand accused before this paper's end.

Thirty years ago the romanticist of the era had an ear tuned weekly to the Wednesday night radio programs where swing and sway music was directed by Sammy Kaye and Rubinoff and his magic violin were noteworthy predecessors to Liberace. A principal source of radio entertainment of the time was listening to the Hit Parade. The Hit Parade featured the ten top tunes of the week and the program had a certain repetitiveness from week to week. As a result, many of the same tunes stayed on the program for a period of several weeks and a length-of-time score was kept on the tunes that persisted in the No. 1 spot. But that was part of the lure and drawing power of this program -- it featured the familiar tunes.

A direct analogy can be drawn between the Hit Parade and a list of microbiological research needs. Research needs, too, have become familiar through time and the priority items have been restated on many occasions. And the needs will not be consumated in the foreseeable future! Research needs generally are couched in language that encourages a seeking of the ultimate. Successful research, to the contrary, results usually in the cohesion of facts for a segment of the whole puzzle -- not in an immediate total solution of the puzzle itself. As facets of a problem become known in our increasingly complex environment, new questions requiring new solutions accelerate clearly into focus. Research proceeds ad infinitum. We need, and we will continue to need, definite answers to particular environmental problems.

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*Director, Technical Support Staff, Office of Air and Water Programs

Needs

Because of the implied interests of the attendees at this seminar, I have limited my research needs remarks to public health-related organisms. I have categorized the research needs proffered into the ten groups following:

1. Detection

I suppose there will be a need always for more refined detection techniques. We need more sensitive rapid quantitative methods to detect pathogenic organisms. In the development of appropriate technology, costs should be held to a minimum, technical training requirements for investigative personnel should be of a reasonable order of magnitude, the time lag in obtaining results should be four hours or less, and procedures should be developed for concentrating viruses and other pathogens from large water volumes. At the present time we are able to recover only a percentage of the viral numbers believed to be within a sample. When a positive answer is obtained from our present detecting procedures, a positive statement can be given; when a negative answer is obtained, there is always an element of doubt. Always it is a search for the elusive few who may be the problem children of their generation.

A corollary concern in detection is media quality control. Specifications for acceptable media are needed. Commercial media now are not getting sufficient quality control and technicians may be forced to rely on media that may be questionable and to produce results that could affect public health. A media certification program might be desirable.

2. Sampling

The time-worn truism is that the results obtained can be no more representative of prevailing conditions than the validity and representability of the sample analyzed. We need to develop and refine techniques to collect large samples from which few organisms may be detected. Bacteriological procedures need to be standardized to become more responsive for enforcement actions. Methods for in situ examination should be developed to complement the need for bench cultures. Automatic monitoring of bacteriological constituents is a need that should receive considered attention. Two ever-present questions are: How many samples do we need? How do we best use these samples in a monitoring program to determine quality of materials sampled?

3. Persistence

Persistence often is a key toward successful mission accomplishment even in the world of the microbes. We need to determine the possible role of water in the transmission of viral diseases from man to man or from animals to man, the degree to which other pathogens are waterborne, and the role of water in the spread of cancer viruses. Do human pathogens decrease in biological wastewater treatment systems at the same relative rate as fecal coliform bacteria? Where sewage-contaminated water is used for crop irrigation, what is the incident and survival of known human and animal pathogens? Such pathogens would include the Mycobacteria on crops irrigated for livestock consumption, various mammalian viruses, as well as the potential for parasitic infestations, including livestock liverflukes or human intestinal parasites.

4. Indication

There is always an element of doubt associated with the use of coliform bacteria as indicators of water quality. Investigations are needed to determine the potential of other organisms either as a virtual replacement of the coliform group or as an adjunct test to more precisely define water quality. A corollary need is better correlation of expected prevalence of indicator and pathogenic organisms in recreational waters. Do human enteric pathogenic organisms increase proportionately to fecal coliform bacteria under environmental conditions where the latter can increase?

5. Disinfection

An ever-present management problem is the adequate disinfection of wastewater effluents. The environmental effects of the present use of chlorine to disinfect wastewaters is of contemporary concern. Viral disinfection and removal capabilities of existing and proposed water and wastewater treatment processes need further evaluation. The effectiveness, efficiency, and economy of disinfection procedures are interrelated factors for consideration in disinfection research. The natural purification processes of surface and ground waters and the effects of nutrients, particularly excess nitrogen and phosphorus on these processes are matters for continuing investigation.

6. Good Looks

There is much circumstantial evidence which indicates that aesthetically pleasing waters generally meet requirements for bacteriological safety. Is it possible to define aesthetic quality to the extent that it could be correlated with bacteriological safety?

7. Sensation

The more specific role of particular microorganisms in imparting organoleptic sensations to water should be determined. There is need for an objective chemical test or tests to identify compounds that cause tastes and odors and to correlate the quantity and quality of such compounds to ambient microorganism populations. Toxicological studies need to determine the relationship between organoleptic sensation-producing organisms and compounds and human health. As discussed under the detection item, it is often necessary to examine large quantities of water in order to identify its taste and odor-producing components. The techniques employed in the collection and examination of mega samples require refinement.

8. Interaction

There are a myriad of interrelationships among the physical, chemical, and biological environments that influence the survival, persistence, and population development of pathogenic and nonpathogenic microbes. The role of various pollutants in these natural phenomena needs more precise definition. Auto-inhibitors or extracellular metabolites that serve as antibiotics to portions of the microbiological community are phenomena related to survival and persistence, which may be quite significant to the potential for disease transmission. The role of sediments and the potential effects of the associated microbiological community on the overlying water is an area that has not received past abundant research. What is the microbial mechanism in both aerobic and anaerobic sediments and the implications to a water supply source, to naturally-occurring shellfish beds or to aquacultural activities? Of a corollary nature, there is a need to establish guidelines to determine the intensities of different types of recreational pursuits that can be sustained concurrently on the same body of water without impairing the relative public use.

9. Risks

Often the professional comment arises that epidemiological data are insufficient for a particular cause-effect relationship. Especially this is true for the transmission of diseases, actual or potential, through recreational-use waters. The eye, ear, nose, and throat infections associated with swimming need a more positive epidemiological correlation with particular water quality. The risks of enteric infection to those associated with water contact sports of varying microbiological water quality should be determined. What is the relationship between the volume of water swallowed, deliberately or accidentally, and the infective dose of pathogenic organisms?

10. Finale

Because of its association with human swimming risks in many nations, amoebic meningoencephalitis, which is thought to be caused by a strain of *Naegleria gruberi*, has stimulated much concern recently. We need to know much more about this organism and its virulent strains. Needs include methods for identifying pathogenic and nonpathogenic forms, additional studies on the organisms' pathogenicity, and the chemical nature of the cytotoxic substance produced by the pathogenic strains.

Although not of the highest priority, there is a need to determine the prevalence in various mammalian and avian populations of pathogenic organisms that could affect man.

Responses

It would be inaccurate to state the EPA research efforts are not attuned to these microbiological problem areas and research needs. Likewise it would be inaccurate to state that the needs in a general sense, will soon be met. They will remain with us, demanding additional research, for many years to come. Research efforts have been chipping away at the visible portion of the iceberg, but they have engaged also in occasional forays into the less evident needs realm. Some of these positive research efforts include the development of:

- Rapid methods for the detection and enumeration of pathogenic bacteria including cultural, biochemical, and serological methods for the evaluation of quantitative and qualitative determinations on Salmonella;

- Improved and more rapid methods for the detection, identification, and enumeration of pollution indicator organisms;

- Methods for the concentration, recovery, and identification of viruses from water;

- Microbiological criteria for recreational waters, for field evaluation of total and fecal coliform and other indicator organisms in marine and estuarine waters;

- Techniques for studying the pathogenic strains of the causative organism of amoebic meningoencephalitis;

- Improved methods of identifying and isolating viruses, identifying diseases transmitted by drinking water, and investigating the endemic occurrence of diseases known to be waterborne.

- Methods for the identification of pathogenic bacteria in rennovated wastewaters and to demonstrate any detrimental effects on human health from water reuse activities;

- Generally applicable chemical and physical treatment methods for the removal or inactivation of microorganisms from municipal wastewaters to any desired degree, which would include a quality suitable for water reuse.

A continuing effort will be devoted to the validation of methods for chemical, biological and microbiological analyses. Efforts to overcome a suppression of coliform organism detection caused by excessive bacterial populations will be researched. The analyses of data indicate that non-coliform bacterial populations in excess of 1,000 Standard Plate Count organisms per 1 ml may affect adversely the detection of the coliform indicator group. This is a problem associated with older water supply distribution networks. The bacteriological quality of bottled water will be examined concurrently to define acceptable criteria. Studies will be made on viral and bacterial pathogens as they occur naturally to determine the influence of environmental conditions, particularly turbidity on disinfection. A corollary problem is to identify acceptable disinfecting procedures without causing consumer rejection of the finished drinking water.

From the above discussion it is apparent that many of the ten itemized research categories are being addressed, in part, through current research. As answers to contemporary questions are found, new questions will arise. Research priorities must be assessed and reevaluated at periodic intervals with an annual appraisal as a minimal goal. Research must continue ad infinitum.

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APPENDIX A

AGENDA

SEMINAR ON STANDARDIZATION OF MICROBIOLOGICAL METHODS

TIME:	January 9-11, 1973
PLACE:	Region IX, San Francisco, California 100 California Street
MODERATOR:	Mr. S. Sid Verner Technical Support Branch Quality Assurance Division Office of Research and Monitoring Headquarters

Tuesday, January 9, 1973

Registration

Welcome - Statement of Problems
Mr. Paul DeFalco, Jr.
Regional Administrator, Region IX

STANDARDIZATION PROCESSES

Enforcement Activities
Mr. David L. Shedroff
Enforcement Division
Office of General Counsel

Research Activities
Dr. Louis G. Swaby
Measurements and Instrumentation Branch
Processes and Effects Division
Office of Research and Monitoring
Headquarters

Regional Activities
Ms. Kathleen Shimmin
Laboratory Support Branch, Region IX

MICROBIOLOGICAL PARAMETERS

Formal presentations will address these topics:

1. General review of method(s) in "Standard Methods"

AGENDA (Continued)

Tuesday, January 9, 1973

2. Available methods as Candidate Methods
3. Suggested recommendations

Discussion: Total Coliforms
Leader: Mr. Harold Jeter
National Training Center, Cincinnati

Discussion: Fecal Coliforms
Leader: Mr. Edwin E. Geldreich
Water Supply Research Laboratory
NERC-Cincinnati

Discussion: Klebsiella
Leader: Dr. Martin D. Knittel
Pacific Northwest Water Laboratory

Wednesday, January 10, 1973

Discussion: Fecal Streptococci
Leader: Mr. Francis T. Brezenski
Technical Support Branch
Region II

Discussion: Viruses
Leader: Dr. Gerald Berg
Virology, AWTRL
NERC-Cincinnati

Discussion: Zoomicrobial Indicators
Leader: Dr. Shih Lu Chang
Water Supply Research Laboratory
NERC-Cincinnati

Discussion: *Pseudomonas aeruginosa*
Leader: Dr. Victor J. Cabelli
Northwest Water Supply Research Laboratory

Discussion: Yeasts, Molds and Fungi
Dr. Leonard J. Guarraia
Office of Water Programs
Headquarters

Thursday, January 11, 1973

Discussion: Salmonella
Leader: Mr. Donald J. Spino
S.W.R. Bio-Engineering and
Science Branch, NERC-Cincinnati

Discussion: Special Problems
Leader: Ms. Kathleen Shimmin
Laboratory Support Branch,
Region IX

PROCEDURES

Formal presentations will address these topics:

1. Current problems and state-of-the-art
2. Need for standardization in sampling and quality control
3. Benefits of standardization in sampling and quality control

Discussion: Sampling
(Collection, Storage,
Chain of custody)
Leader: Mr. William J. Stang
National Field Investigation
Center, Denver

Discussion: Quality Control
(media, equipment, supplies,
performance, data management)
Leader: Mr. Robert H. Bordner
Analytical Quality Control Lab
NERC-Cincinnati

SUMMARIZATION

Research Needs
Mr. Kenneth M. Mackenthun
Direcotr, Technical Support Staff
Office of Air and Water Programs

Adjourn

APPENDIX B

List of Attendees

Name	Office
James H. Adams	Region V, INDO
Elizabeth Anderson	OEGC, Washington, DC
Frederick Au	NERC-Las Vegas, NV
Robert Becker	Water Supply Research Lab. Dauphin Island, AL
G. Berg	NERC-Cincinnati
Bob Bordner	AQCL, Cincinnati
Al Bourquin	GBERL, NERC-Corvallis Gulf Breeze
Francis T. Brezenski	Tech, Support, Region II Edison, NS
Victor Cabelli	NE Water Supply Res. Lab. Naragansett, RI
Bobby J. Carrol	Region IV S/A Athens, GA
Shih Lu Chang	RA Taft NERC-Cincinnati 4676 Columbia PKWY
Howard Davis	NERL, Reg. I, Needham Ht Mass.
William C. Dierkshield	Permits Branch, Reg IX
Reto Engler	Off. Pesticide Prog. Washington, DC
Edwin E. Geldreich	Water Supply Research NERC-Cincinnati
Ronald C. Gordon	AERL, College, AK
Leonard Guarraia	OWP, WQNPS, Washington, DC
Harold Jeter	Water Training, NERC-Cincinnati
Bernard A. Kenner	NERC-Cincinnati
Martin Knittel	NERC-Corvallis
Robert Laidlaw	NFIC-Denver

Name	Office
Ronald F. Lewis	NERC-Cincinnati, AWTRL
Ken Mackenthun	
John P. Manhart	Region VIII, Denver S/A
Robert Manning	NERC-Cincinnati
Robert B. Medz	ORM, Washington, DC
Don Nash	Water Supply Research Lab. Cincinnati
Stephen Poloncsik	ORM, Reg. V, Chicago, IL
Louis A. Resi	NFIC-Cincinnati
William G. Roessler	OPP, Criteria & Evaluation Div., Washington, DC
Robert F. Ruhl	ATB, Washington, DC
Harold Scotten	Region IX, San Francisco
David Shedroff	NFIC-Cincinnati
Kathleen G. Shimmin	Lab. Support Branch, Region IX, Alameda, CA
Andy Sidio	NFIC-Cincinnati
Don Spino	
William J. Stang	NFIC-Denver
G. J. Vascoucelos	NWWSRL, NERC-Cincinnati Gig Harbor, Wash. 98335
Sidney Verner	Office of Monitoring Washington, DC
William H. Winders	Region VI, Enforcement Dallas, TX
S. C. Yin	Robert S. Kerr, Environ., Lab, Ada OK
Ho Lee Young	Lab. Branch, Alameda, Region IX

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